AWARD NUMBER: W81XWH-13-1-0497
PR120718

TITLE: Induction of Food Allergy in Mice by Allergen Inhalation

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Cincinnati, OH 45220-2213

REPORT DATE: December 2016

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
December 2016

2. REPORT TYPE
Final

3. DATES COVERED
30Sep2013 - 29Sep2016

4. TITLE AND SUBTITLE
Induction of Food Allergy in Mice by Allergen Inhalation

5a. CONTRACT NUMBER
W81XWH-13-1-0497

5b. GRANT NUMBER
PR120718

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) Fred Finkelman

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Cincinnati Foundation for Biomedical Research & Education
Cincinnati, OH 45220-2213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal tested a hypothesis about food allergy (FA) pathogenesis. Specifically, we hypothesized that: 1) the route of Ag exposure is critical for determining whether FA development is promoted or suppressed, with airway exposure being more likely than enteric exposure to promote FA; 2) inflammatory costimuli promote the induction of FA by inhaled Ags; 3) inhalation of sub-immunogenic quantities of Ag can induce tolerance instead of priming for FA; and 4) Ag inhalation can sensitize for the development of FA to subsequently ingested, cross-reactive Ags. Using a mouse model of FA to eggs, we found evidence that supports the first 2 hypotheses, with the novel and important observation that saturated fats, including those present in egg yolk, act as an inflammatory costimulus by inducing an unfolded protein response that promotes epithelial cell production of cytokines that stimulate allergy. This led to the clinically relevant observation that FDA-approved drugs that inhibit the unfolded protein response, such as metformin, inhibit the FA induction and suppress established FA. However, studies aimed at demonstrating hypothesis 3 were negative and studies aimed at demonstrating hypothesis 4 were inconclusive.

15. SUBJECT TERMS
Mouse, food allergy, cytokines, eggs, antigen, allergen, inflammation, adjuvant, airways, sensitization

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	UU	86	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION: This proposal tests a hypothesis about the pathogenesis of food allergy (FA): the development of FA depends on a parenteral route of antigen (Ag) exposure, inflammation, Ag dose and Ag epitopes. Specifically, we hypothesized that: 1) the route of Ag exposure is critical for determining whether FA development is promoted or suppressed, with airway exposure being more likely than enteric exposure to promote FA; 2) inflammatory costimuli promote the induction of FA by inhaled Ags; 3) inhalation of sub-immunogenic quantities of Ag can induce tolerance instead of priming for FA; and 4) Ag inhalation can sensitize for the development of FA to subsequently ingested, cross-reactive Ags.

KEYWORDS: Mouse, food allergy, cytokines, eggs, antigen, allergen, inflammation, adjuvant, airways, sensitization

ACCOMPLISHMENTS:

What were the major goals of this project? The major goals of this project, were as stated in the approved SOW (please note that some extend into the third year of the project):

Aim 1: *Determine the conditions under which inhalation of aerosolized egg white can prime for development of food allergy to egg white. Timeframe: months 1-20.*
Task 1. *Determine whether inflammation induced by aspiration of saline would allow exposure to aerosolized egg white to induce allergic airway disease and/or prime for food allergy*
Task 2. Determine whether induction of allergic airway disease and/or priming for food allergy requires airway deposition of a higher dose of egg white than is accomplished by our aerosol protocol.
Task 3. Determine whether induction of allergic airway disease by inhalation of an unrelated allergen will allow exposure to aerosolized egg white (EW) to prime for food allergy (FA).

Aim 2. *Determine whether airway-mediated induction of food allergy by one antigen increases the ability of a second, unrelated antigen to induce food allergy. Timeframe: months 1-12.*

Aim 3. Determine whether ingestion of egg white will inhibit the ability of egg white inhalation to prime for development of egg white food allergy. Timeframe: months 13-24

Aim 4. Test the hypothesis that food regurgitation and aspiration may prime for food allergy. Timeframe: months 13-24.
Task 1: Determine the best time after feeding to recover partially digested egg white from the stomach: Duration: 4 weeks (month 13). Animal requirement: 72 mice.
Task 2: Perform a dose-response study that compares the abilities of fresh egg white vs. stomach-recovered egg white to induce allergic airway disease and initiate food allergy when inoculated intratracheally. Duration: 48 weeks (months 14-24).

Aim 5. Determine whether airway priming with birch pollen can induce murine food allergy to apple and celery. Timeframe: months 25-36.

Aim 6. Determine whether inhalation of aerosolized egg white can reverse

established egg white food allergy. Timeframe: months 1-36.

Task 1: Determine whether inhalation of low doses of aerosolized egg white can suppress established food allergy to this antigen.

Task 2: Histological evaluation of lungs from the same mice used in task 1 to determine effects of the aerosolized egg white on airway inflammation and fibrosis.

Task 3: Produce mAbs to IL-10R, TGF-β and CD25, which will be used in Aim 1, task 2, Aim 3 and Aim 6 task 3. Duration: 36 weeks (months 1-36).

Task 4: Determine whether mAbs to TGF-β, the IL-10R and/or CD25 will block the induction of tolerance by aerosolized egg white.

What was accomplished under these goals?

Aim 1, Task 1: Inducing airway inflammation by causing anesthetized mice to aspirate saline, in addition to having them breathe in aerosolized egg white (EW), still did not cause the development of severe allergic airway disease and sensitization for food allergy to egg white, unlike our original finding with aspiration of EW. The interpretation of this finding was complicated by a failure in many experiments to reproduce our original observation that aspiration of EW-sensitized mice primes mice to develop food allergy, although it always causes some degree of allergic airway disease. Our current interpretation is that two factors are involved: 1) Potentially most important, the presence of some egg yolk acts as an adjuvant for the development of allergic airway disease and food allergy to EW. This is relevant to human allergy, because egg yolk will generally be inhaled along with EW. Please see below for more detail about the adjuvant effect. 2) Our mouse suppliers and some details of the animal husbandry in our mouse colony changed. This has resulted in reduced sensitivity to induction of allergic responses, possibly because of changes in bacterial flora.

Aim 1, Task 2: Increasing the dose of aerosolized EW did not induce allergic airway disease or prime for food allergy. The interpretation of this negative result is complicated by the issues discussed under Aim 1, Task 1 (failure of aspirated EW to prime for food allergy in many experiments).

Aim 1, Task 3: We found that i.t. inoculation of house dust mite extract (HDM) along with EW for the initial 2 inoculations, followed by 17 inoculations (3/week(increases the severity of allergic airway disease that is induced (Figure 1). This is potentially important,

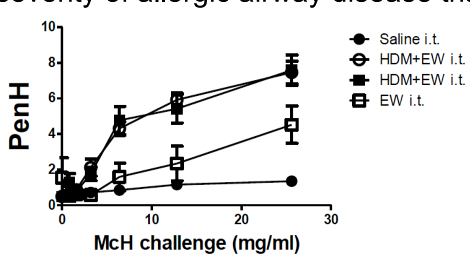

Figure 1. Inhalation of HDM with EW increases allergic airway disease beyond that induced by EW alone. BALB/c mice were inoculated i.t. with saline, EW, or HDM + EW for the first two inoculations, then with EW for 17 additional inoculations. Two separate groups of mice were inoculated with HDM + EW, to test reproducibility. Mice were tested by barometric plethysmography for responsiveness to mechacholine.

because house dust in most of the US typically contains egg proteins as well as house dust mites; consequently, it is likely that this combination of antigens will be inhaled. Studies have not been performed to determine whether the initial HDM inoculation with EW makes mice susceptibility to develop EW food allergy. This is because of the stronger effect that we later found of egg yolk plasma (EYP, the liquid part of egg yolk) on the development of both allergic airway disease and food allergy to EW (Figure 2; food allergy is detected as diarrhea and anaphylactic shock, which is observed as

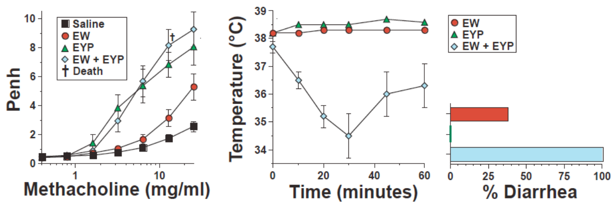

Figure 2. EYP enhances development of food allergy to EW. BALB/c mice were inoculated i.t. 3x/wk x 3 wks with saline, EW, EYP or EYP + EW, then tested for airway responsiveness to methacholine (left panel), after which they were inoculated o.g. 2x/wk x 4 wks with the same Ags and evaluated for development of shock (hypothermia) and diarrhea.

hypothermia. We have not been able to test whether inhalation of an aerosol that contains both EW and egg yolk will induce severe allergic airway disease and prime for development of egg food allergy, because the high viscosity of egg yolk makes aerosol generation difficult with our equipment. We have found that a low concentration of egg yolk (nanogram range) still has an adjuvant effect when applied with egg white to the skin, but we have not tested this in the airway priming model.

The strong effect of EYP on the development of airway hyperresponsiveness (AHR) and the strong synergy between EW and EYP on the development of food allergy had obvious human relevance, because EW and egg yolk (EY) are likely to be encountered together by humans, but was somewhat surprising, because the most clinically important egg allergens are present in EW, rather than EY. This, and the predominantly lipid constitution of EYP, made us wonder if EYP promoted airway and food allergy to eggs by virtue of possible adjuvant effects of its lipid components, rather than antigenic effects of its protein components. The former possibility seemed feasible, because we had recently found and reported that saturated fats, including those that are present in EYP, can induce epithelial cells to produce TLSP, IL-25 and IL-33, cytokines that have been shown to be important for the development of a food allergic response. To test this possibility, we evaluated whether EYP could induce epithelial cell expression of TSLP, IL-25 and/or IL-33 whether applied for 24 hours to mouse skin or inoculated i.t. 24 hours prior to harvesting mouse lungs. Results of this experiment (Figure 3) showed

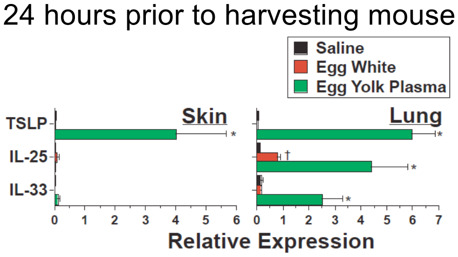

Figure 3. EYP induces pro-Th2 cytokine responses in vivo. BALB/c mice (6/gp) were inoculated i.t. with saline, EW, or EYP or had these applied to unabraded skin under an occlusive dressing. 24 hr later RNA was extracted from skin and lungs and relative expression of TSLP, IL-25 and IL-33 genes was determined by quantitative RT-PCR. * indicates a significant ($p<0.05$) difference from saline and EW-treated mice; † indicates a significant difference from saline-treated mice.

that EYP strongly induced TSLP expression when applied to skin and all 3 of these cytokines when inoculated into the lungs. In contrast, EW has little ability to induce these cytokines. To determine whether induction of these cytokines was relevant, we first tested whether they are important for induction and maintenance of FA in another, more established model, in which food allergy is induced by inoculation with EW + purified saturated medium chain triglycerides by oral gavage (o.g.). Our results show that all three of these cytokines, which we call “pro-Th2 cytokines” because they promote a Th2 cytokine response, are essential for induction of food allergy in this model (Figure 4), while any one of these pro-Th2 cytokines can maintain established food allergy (Figure 5). These observations both explain how eggs (and other important nutrients that have a high saturated fat content, such as cow’s milk), can be such common and important food allergens and provide an approach for the suppression of established FA.

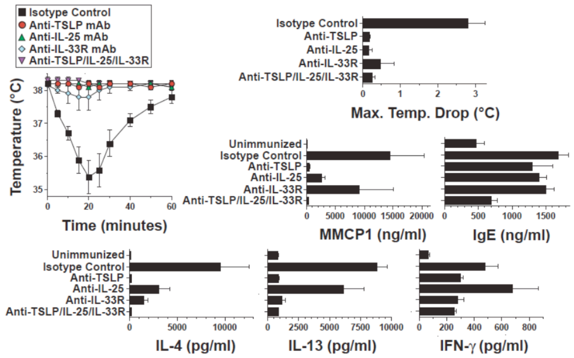

Figure 4. Anti-TSLP, anti-IL-25 and anti-IL-33R mAbs suppress food allergy induction by MCT + EW. BALB/c mice (6/group) were left untreated or were inoculated o.g. 3x a week for 3 weeks with 100 mg of EW + 100 µl of MCT. Mice were also injected i.p. 12 hr before each o.g. inoculation with mAbs to TSLP (500 µg), IL-25 (500 µg), or IL-33 receptor (IL-33R, 100 µg), with a combination of all 3 mAbs, or with isotype control mAbs. Mice were evaluated for development of hypothermia and MMCP1, IL-4 and IL-13 responses to their final o.g. challenge with 100 µl of MCT + 100 mg of EW.

These observations provided the basis for a paper that was submitted for publication to the *Journal of Allergy and Clinical Immunology*. The paper received a favorable review and is now being re-reviewed by the same journal after it was revised in response to the suggestions of the reviewers.

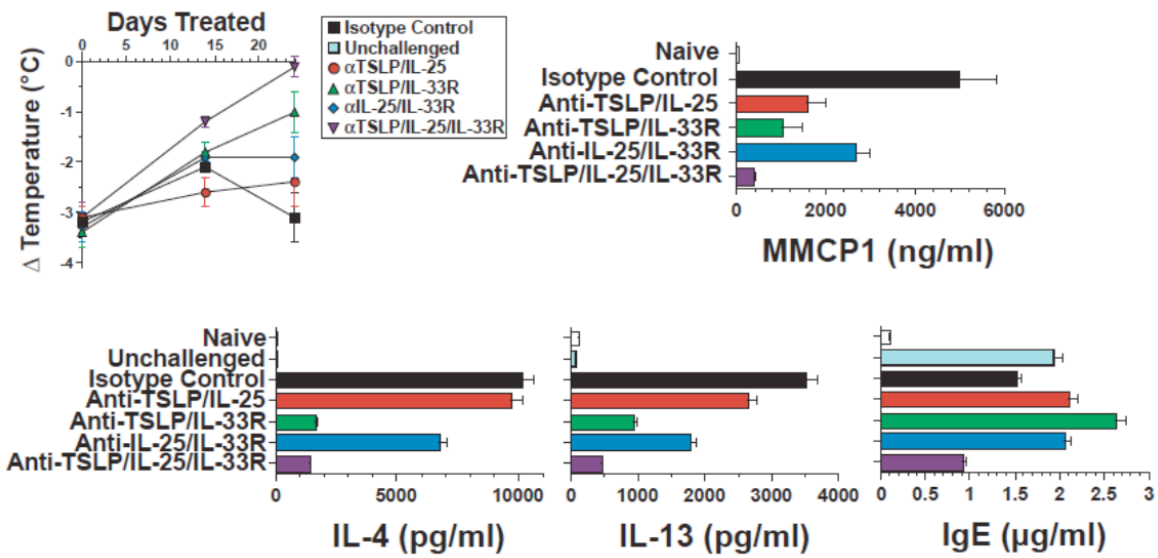

Figure 5. Suppression of established FA with anti-pro-Th2 mAbs. BALB/c mice were inoculated o.g. twice with MCT, then 2x/wk for 4 wk with MCT/EW. Mice with a temperature drop >3°C in response to o.g. challenge were then divided into groups of 5. These mice continued to be inoculated o.g. 2x/wk with MCT/EW for an additional 24 d, but also were injected i.p. with the mAbs shown 12 hr before each o.g. inoculation. Maximal decreases in rectal temperature were determined for the hr after o.g. inoculation on d 0, 14 and 24 after initiation of mAb treatment. Serum IgE and MMCP1 levels and IL-4 and IL-13 production were determined on d 24.

We then repeated the observations illustrated in Figure 5, using sensitization by the intratracheal (i.t.) route with EW + EYP instead of o.g. sensitization with EW + MCT. Our results (Figure 6), demonstrated that FA induced by i.t. priming with EW + EYP, followed by oral gavage with EW + EYP, can also be suppressed by treatment with a combination of mAbs to IL-25, the IL-33R, and TSLP. This observation has practical importance, because, combined with the data in Figure 5, it provides evidence that agents that suppress IL-25, IL-33, and TSLP production or effects might be useful to prevent and even treat FA. This practical importance is illustrated by novel observations made by my group with funding from other sources. Specifically, we found that drugs that suppress the unfolded protein response such as metformin, also suppress EYP

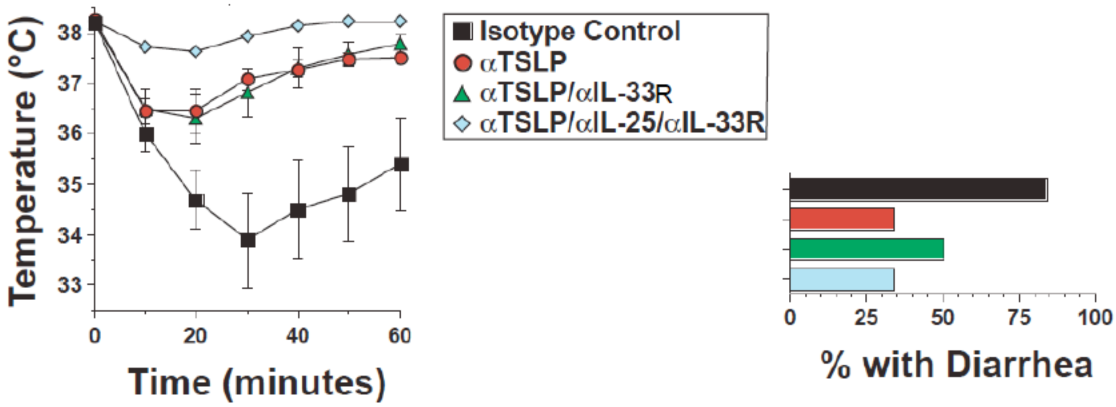

Figure 6. Murine FA induced by i.t. priming with EYP + EW, followed by oral gavage with the same foods, is suppressed by treatment with monoclonal antibodies to IL-25, IL-33R and TSLP. FA was induced in BALB/c mice by i.t. priming, followed by oral gavage, as in Fig. 2. When mice had developed hypothermia in response to oral gavage (o.g.) with EW + EYP, they continued to be inoculated o.g. with these foods, but also were treated with monoclonal antibodies to TSLP, IL-33R, and/or IL-25, as in Fig. 5. Mice were evaluated for the development of diarrhea and/or hypothermia in response to oral gavage with EW + EYP after 5 weeks of monoclonal antibody treatment.

induction of IL-25, IL-33 and TSLP responses. This observation, in turn led us to test whether metformin (an FDA-approved drug that is widely used to treat diabetes mellitus and is relatively safe, could prevent FA and/or suppress established FA. Our preliminary data (Figure 7) suggest that this is the case. This suggests that metformin might be

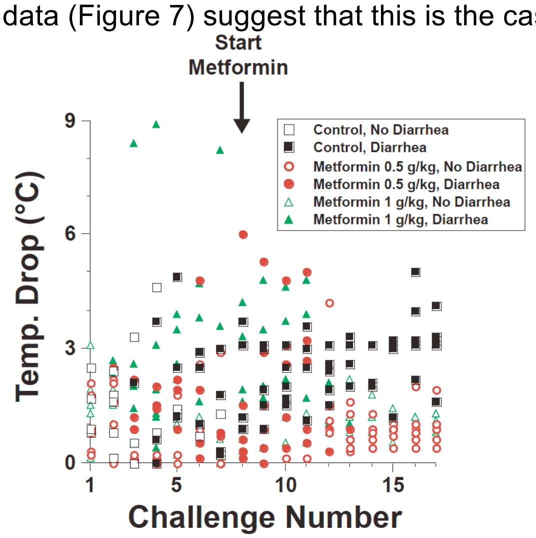

Figure 7. Metformin can suppress established FA in mice. BALB/c mice (5-6/group) were sensitized transcutaneously to EW + EYP, then inoculated by o.g. with EW + EYP 2x/week until all developed diarrhea and most developed hypothermia in response to oral gavage with these foods. Mice were then supplied with normal drinking water or drinking water that provided approximately 0.5 grams/kg of metformin/day or 1 gram/kg of metformin/day. Mice continued to be inoculated o.g. 2x/week with EW + EYP. Although this experiment is still in progress, note that both doses of metformin completely suppressed the diarrheal response and considerably suppressed the hypothermia response.

useful to treat human FA. We have applied for funding to pursue this possibility. Although these studies were not strictly encompassed by our DOD award and were funded through other sources, they would not have been performed without the observations made in our DOD-supported studies.

<u>Aim 2:</u> We have induced allergic airway disease and subsequently, food allergy, to EW and then evaluated whether ingestion of an aqueous peanut extract would induce peanut food allergy. The results were negative, although we cannot exclude the possibility that more intense food allergy to EW or a different immunization schedule with peanut extract would have allowed induction of food allergy to peanut. This is suggested by our collaborative studies with Dr. Xiu-Min Li at Mt. Sinai College of Medicine, that have shown that it takes longer to induce peanut allergy than egg allergy when mice are primed with either food by transcutaneous sensitization.

Aim 3: An initial experiment suggested that ingestion of EW suppresses the ability of aspirated EW to induce allergic airway disease. However, a second experiment that used a larger number of mice and a more rigorous approach to detect allergic airway disease (measurement of resistance by an invasive technique with a flexiVent apparatus) gave the opposite result. This observation may be clinically significant because it indicates that inhalation of egg might overcome oral tolerance. This could explain how adults, who have eaten eggs many times, can develop food allergy to eggs.

Aim 4, Tasks 1 and 2: These tasks were not completed. This is because our observations with mixtures of EW + EYP indicate that acidification/partial digestion is not necessary for airway sensitization to prime for food allergy.

Aim 5: We inoculated mice i.t. with both crude birch pollen and a commercial birch pollen extract. Neither stimulated airway hyperresponsiveness to methacholine or sensitized mice to develop food allergy to celery or apple. This suggests that either the BALB/c mouse is not an appropriate species to use to model this or that stimuli in addition to airway inoculation with birch pollen are required.

Aim 6, Task 1: Mice were induced to develop allergic airway disease to EW by airway inoculation with HDM + EW, followed by EW, as in Figure 1, or were inoculated i.t. with saline (negative control). Mice were then were exposed to aerosolized EW or bovine serum albumin (BSA, negative control) 3x/week for 4 weeks, with the expectation that the relevant antigen (EW) might suppress airway hyperresponsiveness more than the irrelevant antigen (BSA). Instead, if anything, the EW aerosol acted to maintain airway responsiveness (Figure 8). This suggests that a low dose of an aerosolized

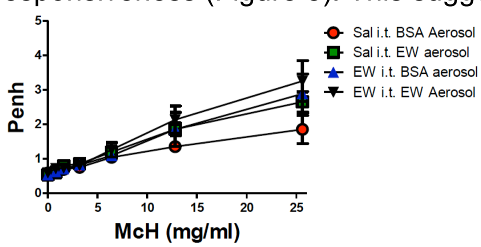

Figure 8. Inhalation of aerosolized EW fails to specifically suppress airway hyperresponsiveness in EW immunized mice. Mice were immunized i.t. to EW as in Figure 1, or were inoculated i.t. with saline, then were exposed 3x/wk for 4 wk to aerosolized EW or BSA. After this, they were evaluated for responsiveness to aerosolized methacholine.

antigen is not effective at suppressing established allergic airway disease to that antigen.

Aim 6, Task 2: This was not performed because of the negative results of Task 1.

Aim 6, Task 3: All of the mAbs were prepared.

Aim 6, Task 4: The planned study cannot be performed because aerosolized EW failed to induce tolerance in EW-immune mice. Instead, with permission of our Scientific Officer, we followed up on the most exciting results of our study, those of Aim 1, Task 3, and determined the mechanism by which EYP promotes allergic airway disease and food allergy to EW (i.e.; it stimulates the unfolded protein response in epithelial cells (in airway, skin, and gut), which promotes the production of IL-25, IL-33 and TSLP by these cells). Further work on this aspect of our project was cut short when the Cincinnati VA's non-profit corporation, CERV, refused to support an extension without additional funding.

Opportunities for training and professional development: One post-doctoral fellow, Durga Krishnamurthy, was hired under this contract. I have met with her at least

weekly to discuss results and plan additional experiments throughout the period of this contract, with the exception of her annual leave time and the 4 months following her complicated delivery.

Dissemination of results: Abstracts were presented at the American Association of Immunology Annual meeting by Dr. Marat Khodoun and Dr. Unni Samavedam that describe our results. The former was chosen for both oral and poster presentation; the latter for poster presentation. The latter received an award for exceptional merit. I presented our data at a FA symposium at Harvard University. I have been asked not to present our metformin data until our position with regard to intellectual property is more established. As noted earlier, a paper that describes some of our data is in the second round of review at the *Journal of Allergy and Clinical Immunology*.

Plans for the next reporting period: Nothing to report.

IMPACT:

Impact on the principal discipline: The principal impact was 4-fold:

1. We demonstrated that inhalation of egg white plus egg yolk can sensitize to allow the development of food allergy in response to ingested EW + EY. This demonstrates an alternative pathway, aside from skin sensitization, that can prime for the development of egg allergy, one of the most common food allergies.
2. We demonstrated synergy between egg white and egg yolk in the induction of allergic airway disease and food allergy. This suggests that the two component of eggs have distinct roles in the induction of egg allergy and that the use of egg white as a nutrient, without egg yolk (or with an unsaturated fat substituting for egg yolk) might decrease the prevalence of egg allergy.
3. We demonstrated that egg yolk, but not egg white, induces lung and skin epithelial cells to express three cytokines (hormones of the immune system) that promote the development of food allergy. We showed that all three of these cytokines are required to induce food allergy in our model, while any one will maintain established food allergy. This suggests that an approach that neutralizes all 3 of these cytokines or inhibits their production may be necessary to suppress established food allergy.
4. Our observations that the unfolded protein response is an important intermediate to production of IL-25, IL-33 and TSLP and that metformin, an inhibitor of the unfolded protein response, can suppress EYP induction of IL-25, IL-33 and TSLP and suppress establish food allergy has the potential to change the way that this disease is treated.

Impact on other disciplines: The discovery that egg yolk plasma, and other saturated fats, may act as Th2 adjuvants may provide a relatively safe and useful adjuvant to use for vaccination.

Impact on technology transfer: Nothing to report

Impact on society beyond science and technology: As noted above, our discovery that metformin can suppress established food allergy has the potential to improve the treatment of this disorder and possibly other allergic diseases as well.

CHANGES/PROBLEMS:

Changes in Approach: Nothing to report

Delays: A significant delay was caused by pregnancy/delivery-related problems, followed by child-care problems, of the post-doctoral fellow who is doing a great deal of the work on this project. We were never able to fully catch up and had to prioritize the experiments that were performed.

Changes in expenditures: Nothing to report

Changes in human subjects, vertebrate animals, biohazards/select agents: Nothing to report.

PRODUCTS:

Publications:

1. Marat Khodoun, Durga Krishnamurthy, Richard Strait, Joel Tocker, and Fred D. Finkelman. Suppression of established food allergy by a combination of anti-TSLP, anti-IL-33, and anti-IL-25 monoclonal antibodies. (Abstract). *J. Immunol.* 196 (1 Supplement), 123.5
2. Unni K. Samavedam, Marat Khodoun M, David Wu, Simon P .Hogan, and Fred D. Finkelman. Saturated fatty acids promote allergic (Th2) cytokine responses by activation of unfolded protein response (UPR) and ER stress. Abstract). *J. Immunol.* 196 (1 Supplement), 123.9.
3. Khodoun, MV, Tomar S, Tocker JE, Wang Y-H, Finkelman, FD. Prevention of food allergy development and suppression of established food allergy by neutralization of TSLP, IL-25 and IL-33. *J Allergy Clin Immunol*. In second round of review.
4. Unfolded protein response suppression as a treatment for murine food allergy. Presented at Harvard Food Allergy Conference, December, 2016.

Other products: An invention disclosure is being prepared regarding the potential use of unfolded protein response inhibitors, including metformin, in the treatment of food allergy and other allergic disorders.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

Name: Fred Finkelman, M.D.
Project Role: PI
Researcher identifier:
Nearest person months worked: 3.0/year x 3 years = 9
Contribution to Project: Directs project; plans experiments, interprets results, writes papers and reports.
Funding Support: This grant

Marat Khodoun, Ph.D.
Project Role: co-Investigator
Researcher identifier:
Nearest person months worked: 3.72/year x 3 years = 11.16
Contribution to Project: Prepares reagents, inoculates and tests mice for food allergy; contributes to planning and interpreting experiments.
Funding Support: This grant

Durga Krishnamurthy, Ph.D.
Project Role: co-Investigator
Researcher identifier:
Nearest person months worked: 10.0/year x 2.5 years = 25
Contribution to Project: Inoculates and tests mice for food allergy; evaluates mice for tolerance induction; contributes to planning and interpreting experiments.
Funding Support: This grant.

Charles Perkins, B.A.
Project Role: Research Assistant
Researcher identifier:

Nearest person months worked: 12.0/year x 2.83 years = 33.96
Contribution to Project: Inoculates mice intratracheally and performs studies of lung function, assists with studies of intestinal function; prepares monoclonal antibodies.
Funding Support: This grant

Crystal Potter, B.A.
Project Role: Research Assistant
Researcher identifier:
Nearest person months worked: 3.0/year x 3 years = 9
Contribution to Project: Breeds and PCR types mice
Funding Support: This grant

Changes in active other support of the PI and senior/key personnel since the last reporting period.

None to report.

Other organizations involved as partners: None to report.

8. Special reporting requirements: None

9. Appendices: Khodoun, MV, Tomar S, Tocker JE, Wang Y-H, Finkelman, FD. Prevention of food allergy development and suppression of established food allergy by neutralization of TSLP, IL-25 and IL-33. *J Allergy Clin Immunol*. In second round of review.

Elsevier Editorial System(tm) for Journal of Allergy and Clinical Immunology
Manuscript Draft

Manuscript Number: JACI-D-16-00800R1

Title: Prevention of food allergy development and suppression of established food allergy by neutralization of TSLP, IL-25 and IL-33

Article Type: Original Article

Section/Category: Food, Drug, and Insect Sting Allergy and Anaphylaxis

Keywords: cytokines; food allergy; IgE; mast cell; therapy; anaphylaxis; IL-25; IL-33; TSLP

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Manuscript Region of Origin: USA

Abstract: Background: Food allergy (FA) is an increasing problem that has no approved treatment. The pro-Th2 cytokines, IL-25, IL-33 and TSLP, are associated with FA and monoclonal antibodies (mAbs) to these cytokines are reported to suppress murine FA development.
Objective: Determine whether anti-pro-Th2 cytokine mAbs can block both FA maintenance and induction.
Methods: IgE-mediated FA was induced in BALB/c mice by oral gavage (o.g.) with medium chain triglycerides plus egg white (MCT/EW) and was characterized by increased numbers of lamina propria Th2 cells, mast cells shock, and eosinophils, shock (hypothermia), mast cell degranulation (increased serum MMCP1), increased serum IgG1 anti-EW and IgE, and increased IL-4 and IL-13 secretion following MCT/EW challenge. To suppress FA development, mice were injected with anti-IL-25, IL-33R, and/or TSLP monoclonal antibodies prior to the initial o. g. with MCT/EW; to suppress established FA, treatment with the same mAbs was initiated after FA development.
Results: Injection of a mAb to IL-25, IL-33R, or TSLP strongly inhibited FA development. No single mAb to a pro-Th2 cytokine could suppress established FA and optimal FA suppression required treatment with a cocktail of all three anti-pro-Th2 mAbs. Treatment with the three mAb cocktail during initial MCT/EW immunization induced EW tolerance.
Conclusion: All of the pro-Th2 cytokines are required to induce our model of FA, while any pro-Th2 cytokine can maintain established FA. Pro-Th2 cytokines prevent oral tolerance. Combined treatment with antagonists to all three pro-Th2 cytokines or with an inhibitor of pro-Th2 cytokine production may be able to suppress established human FA.

To the Editors:

My colleagues and I were glad to receive the review of our manuscript, JACI-D-00800, Prevention of food allergy development and suppression of established food allergy by neutralization of TSLP, IL-25 and IL-33

Our paper made two points that we thought were both quite novel and important. We provided the first evidence, in any mouse model, that established that food allergy could be reversed by neutralizing cytokines. We demonstrated that this could be accomplished by a combination of anti-IL-25, anti-IL-33 ligand, and anti-TSLP monoclonal antibodies, while any one or two of these antibodies could not fully reverse established disease. We also showed that the cytokine requirements for suppressing established disease differ considerably from the requirements for inducing disease, inasmuch as a monoclonal antibody to any one of these cytokines prevented food allergy induction in our model. Furthermore, we showed that blocking these cytokines during immunization led to tolerance induction in our model; this indicated that the three cytokines that we studied convert a tolerogenic stimulus to an immunogenic one.

These results were not at all obvious to us when we initiated our study. Two of your three reviewers seemed to agree, inasmuch as they discussed the importance of our work. Reviewer #1, however, characterized our work as incremental, suggesting that it was a small addition to what was already known. We think this opinion is difficult to justify, because we are unaware of any previous data that would have predicted our results. We think that the obvious clinical implications of our observations make them biologically and medically important.

We are limited in our response to the Reviewers because the Janssen subsidiary of Johnson & Johnson, the source of our anti-IL-25 and anti-IL-33 ligand monoclonal antibodies, lost interest in this area and is no longer able to provide the antibodies and unwilling to provide the hybridomas. We had sufficient quantities of these antibodies for only one additional experiment. Because we agreed with Reviewer #4 that it would be useful to know how combined treatment with anti-IL-25, anti-IL-33 ligand and anti-TSLP monoclonal antibodies affects Th2 cells, ILC2s, dendritic cells, basophils, and mast cells in the intestinal lamina propria and mesenteric lymph node of mice that had established food allergy, we used our remaining antibody for this purpose. We have also responded positively to all of the other comments of Reviewer s #2 and #4, with the exception of Reviewer #4's suggestion about transferring antigen-primed Th2 cells (the results of our new experiment decreased the relevance of this suggestion to the points that we are trying to make).

We were not, however, able to do the studies suggested by Reviewer #1 because of the lack of the required antibodies. Further, although the results of the suggested studies of the effects of each anti-cytokine antibody on intestinal cell types and of the mechanisms involved in oral tolerance in our model would certainly be of interest, they would likely take over a year to perform, even if we had the antibodies

to perform them. This is longer than we think publication of our important results should be delayed.

As you know, we wrote to you about these concerns before deciding which experiments to perform and whether to resubmit a revised paper to the JACI. In response we received the following message: “I think that you should definitely submit your revised manuscript to JACI. Given the reasons that you have outlined in your letter - and the fact that this work is of great interest to the Journal - I do not think that the concerns of reviewer #1 will end up being a deal breaker.”

As a result, we preformed the additional study referred to earlier, as well as an experiment that shows that the development of hypothermia in our model is IgE-mediated. Our point-by-point responses to the Reviewers follow (changes on the marked version of our manuscript are shown in red type):

Reviewer #1:
1. While these studies are interesting and contain useful information regarding the potential use of individual antibodies against IL-25, IL-33, and TSLP cytokine systems, they do not extend into how each individually or together are altering the responses. As I've outlined in the review, the studies need to extend these observations into trying to assess the cellular responses as well as assessment of the disease model itself. This is important. Otherwise, I feel that these studies are quite incremental without additional significant insight.

In response to the Reviewer, we have performed an experiment (Fig. 5) that shows that development of food allergy in our model is accompanied by substantial increases in Th2 cells and mast cells as well as some increases in eosinophils and DCs in the lamina propria, with no significant increase in lamina propria ILC2s as conventionally defined and no significant increase in any mesenteric lymph node cell population. We also show that these changes, except for the increase in DCs, are reversed by treatment with a cocktail of antibodies to IL-25, IL-33 ligand and TSLP. However, we respectfully disagree with the characterization of our studies as “quite incremental,” inasmuch as they provide the first indication that the pro-Th2 cytokines are important for maintenance of food allergy. This will come as a surprise to those who have suggested that the main role of these cytokines is the *initiation* of a type 2 cytokine (IL-4/IL-5/IL-9/IL-13) response. In addition, to the best of our knowledge, our results provide the first example of reversal of established food allergy by cytokine suppressive therapy. We have previously published in the JACI, for example, that even a combination of anti-IL-4 receptor ⍰ mAb and anti-IL-4 mAb did not have this effect (Brandt, E. B., A. Munitz, T. Orekov, M. K. Mingler, M. McBride, F. D. Finkelman and M. E. Rothenberg. 2009. Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea*. J All Clin Immunol.* 123:53-8). As such, we believe that these results are an important step forward from both a basic and a clinical standpoint.

2. In these studies the data indicate that the combination of antibodies against IL-25, TSLP and IL-33R may be useful for ongoing allergic disease induced by food. The studies initially examined the role of each cytokine on its own by neutralizing from the onset of disease sensitization. Indeed, neutralization of any one of the cytokines was important for development of disease as indicated by temperature drop, an important aspect of disease. However, other clinical parameters should also be characterized. While these data highlight that it is unlikely that targeting a single innate cytokine pathway, IL-25, TSLP, or IL-33, on its own will be useful, the data do not further characterize the potential mechanism of the effect. Thus, a number of interesting data may provide greater insight and impact for the publication.

Are there differential cellular effects that each of the antibody treatments provide that result in the decreased disease phenotype in the studies in Figure 1? This may be important to understand and compare. For instance, anti-IL-33R appears to be distinct from anti-TSLP and anti-IL-25, based upon the MMCP-1 levels. Additionally, the differential regulation of cytokines also indicated individual changes. The accumulation and expansion of ILC-2s, basophils, mast cells, etc. each could be differentially altered by the neutralization of each of the cytokine systems. These data would give important information and greatly add to the understanding of the mechanism.

We agree that it would be useful to look individually at the effect of inhibition of each pro-Th2 cytokine. Unfortunately, Janssen, which provided us with the anti-IL-33R and anti-IL-25 mAbs that we have used, has abandoned research that is directed towards these potential therapeutic targets. Janssen is, consequently, no longer able to supply us with these mAbs, and is unwilling to provide us with the hybridomas. Although there are some commercial sources of these mAbs, the cost would be too great for us to purchase them for the 3-5 week courses of treatment that have proven necessary. That said, the one additional study that we were able to perform did eliminate ILC2 expansion as a mechanism of food allergy development in our model, because there is no increase in ILC2s. Based on the large increases in Th2 cells and mast cells, the reversal of these increases with combined anti-pro-Th2 cytokine treatment, evidence that each of the pro-Th2 cytokines can promote a Th2 cell response, and evidence that Th2 cytokines can promote a mast cell response, we favor the view that the most important combined effect of the pro-Th2 cytokines is promotion of a Th2 response, followed by IgE secretion and Th2 plus pro-Th2 cytokine-induced expansion of the mast cell response. Although previous studies of intestinal worm infection models demonstrated large increases in intestinal ILC2s, a previously published study of murine food allergy showed that murine food allergy models in which priming was accomplished by i.p. immunization with OVA/alum or skin sensitization with OVA/vitamin D also demonstrated increases in mast cells and Th2 cells, but not ILC2s, in the lamina propria (Chen, C. Y., Lee, J. B., Liu, B., Ohta, S., Wang, P. Y., Kartashov, A. V., Mugge, L., Abonia, J. P., Barski, A., Izuhara, K., Rothenberg, M. E., Finkelman, F. D., Hogan, S. P., Wang, Y. H. 2015. Induction of interleukin-9-producing mucosal mast cells promotes susceptibility to IgE-mediated experimental food allergy. *Immunity*. 2015. 43:788-802 – see supplemental figures).

Thus, Th2 cells appear to be more important than ILC2s for the type 2 cytokine responses in murine food allergy.

3. The ability to induce tolerance is very interesting. However, no mechanism is explored. Is the tolerance due to the generation of Treg cells, reduction in cellular recruitment, decreased ILC2 cells, etc. ? These data would greatly enhance these observations.

Again, the nonavailability of critical reagents has kept us from studying this further, although, as noted above, our data make it unlikely that suppression of an ILC2 response has a critical role.

4. In all of the studies the data rely on temperature drop as the single clinical disease parameter for determining if there was an effect or not. While this is an important measurement, there have been several others established in the literature, including gut leak, development of diarrhea, and a clinical scoring system. Furthermore, histologic assessment would be important to understand whether the effect of the treatment was protective to the gut epithelium and/or reduced inflammation in each treatment. These are all important given the differential effects that were observed on cytokines, IgE, and temperature during the studies in the different neutralization strategies.

We have previously reported that there is a close correlation between temperature drop and a clinical score that is based predominantly on changes in movement and ability to remain erect (Strait, R., S.C. Morris, M. Yang, X.-W. Qu, and F.D. Finkelman. 2002. Pathways of anaphylaxis in the mouse. *J. All. Clin. Immunol.* 109:658-68). We have also previously reported that temperature drop in our anaphylaxis models is based predominantly on an increase is vascular leak (with hemoconcentration that correlates well with temperature drop (Strait, R.T., Morris, S.C., Smiley, K., Urban, J.F.Jr., and Finkelman, F.D. 2003. IL-4 exacerbates anaphylaxis. *J. Immunol.* 170:3835-42)). Because diarrhea is not a feature of the food allergy model that we have studied, increased gut leak is either not prominent or is compensated for by increased reabsorption of fluid from the gut. Again, the nonavailability of reagents prevents us from studying this further.

REVIEWER #3:

Comments on the manuscript entitled: "Prevention of food allergy development and suppression of established food allergy by neutralization of TSLP, IL-25 and IL-33" by Khodoun MV, Tocker JE and Finkelman FD.

Summary:

In this manuscript, Khodoun VM et al. used neutralizing antibodies prior to initiation of an established model of food allergy in mice to demonstrate that all pro-Th2

cytokines (TSLP, IL-33 and IL-25) are required for development of the disorder. Moreover, the combined treatment of neutralizing antibodies against pro-Th2 cytokines can suppress established food allergy.

Major comments:

The authors show strong evidence that the combined use of neutralizing antibodies against pro-Th2 cytokines can prevent the development or suppress established food allergies.

My major comments are the following:

1-The statistics in the figures are missing; therefore, it is very difficult to assess the significance of the data.

We apologize for not having done a better job with statistical analysis of our data. We have now consulted with a statistician and added these data, which demonstrate that our key observations are significant. Our methods for statistical analysis are described in the Statistics section of Materials and Methods.

2-It is unclear to this reviewer whether the outcomes of the FA protocol used match those described in a previous publication by the same group (ref. 29). The IgE levels immediately post-treatment seem to be significantly lower than 5 weeks post treatment for the control antibodies. However, the MMCP1 levels are reduced following the neutralizing antibody treatment. Is it possible that mast cell activation is IgG dependent rather than IgE dependent after three weeks of treatment with MCT/EW? This needs to be clarified.

We appreciate this helpful question. To answer it, following the development of food allergy in response to MCT/EW, we treated mice with either EM-95 (rat IgG2a anti-mouse IgE mAb), to block IgE-mediated anaphylaxis, or 2.4G2 (rat IgG2b anti-mouse FcγRIIB/RIII) mAb, to block IgG-mediated anaphylaxis. Our results, included as supplemental figure 1, demonstrate that the temperature drop caused by MCT/EW challenge one day after EM-95 or 2.4G2 treatment is IgE-, not IgG-dependent (i.e.; it is blocked completely by pre-treatment with EM-95 but not significantly by pre-treatment with 2.4G2). This result is similar to the observation made in our reference 29 when mice were challenged o.g. with allergen.

3-The IgG1 data are only shown in Fig. 1 but not in the rest of the figures. Can you please indicate why?

We have added IgG1 data to the other figures. Our data show that only the combination of anti-TSLP, anti-IL-25 and anti-IL-33 mAbs suppressed IgG1 levels and that suppression by this mAb combination was approximately 50%.

4-Figure legend 2 indicates that a separate group of mice continued to be treated with

MCT/EW after the 3 week treatment. Can you please indicate for how long the mice were treated in Fig. 2?

We agree that the legend for Figure 2 was confusing. We have re-written it and now include a diagram of the protocol in that figure that makes clear that mice were treated with MCT for 3 days, followed by MCT/EW plus isotype control or one or more anti-pro-Th2 cytokine mAb for an additional 3 weeks.

5-In lines 160-161, the authors indicate that each of the anti-pro-Th2 mAbs suppressed the MMCP1, IL-4 and IL-13 responses to oral challenge. However, it seems to me that anti-IL-33R did not suppress MMCP1 release and anti-IL-25 did not suppress IL-13 release.

Thank you for pointing this out. We have now added the statistical analysis and more specifically note the effects of the different anti-pro-Th2 cytokine treatments.

6-It is difficult to assess without the stats, but my impression is that anti-TSLP, anti-IL-33R and their combination did partially suppress IFN-g release in Fig. 2. This needs to be clarified and or corrected in the statement in lines 169-171.

We have now added statistical analysis and indicate in Fig. 2 that the changes in IFN-γ secretion were not significant.

7-This reviewer finds it difficult to understand whether there is a difference in the FA protocols used in Figs. 3 and 4, which prevents combining the figures. Also, the titles for the figure legends are basically the same.

Although the experiments shown in Figs. 3 and 4 had a similar design, they differed in the combinations of anti-pro-Th2 mAbs that were used, in the precise protocols used to induce and to treat food allergy and in that the results of anti-pro-Th2 mAb treatment were evaluated at 2 timepoints in Fig. 4, but at only 1 timepoint in Fig. 3. For these reasons, we think it is best to keep the figures separate. The differences in the protocols should now be apparent from the diagrams shown at the top of each figure.

Minor recommendations:

1-Stats: Mann-Whitney is not a t-test.

Thank you for pointing this out. We apologize for the problems with our statistical analyses and their descriptions. These have been corrected. Changes are described in the Statistics section of Materials and Methods.

2-Schematics showing the protocols for inoculation and antibody administration may help the reviewers to understand the data relevance. As an example, it is difficult to

understand what the authors refer to as "pre-challenge or post-challenge" in Fig. 3 or "Immediately post-treatment" and "5 weeks post treatment" in Fig. 1.

We appreciate this suggestion and have now diagrammed the protocol used at the top of each figure.

REVIEWER #4:

This is a very interesting manuscript from Kordoun et al describing the role of the epithelial cytokines TSLP, IL-25, and IL-33 in a model of food allergy. They show that blockade of these cytokines during either sensitization or after establishment of disease. For the former set of studies blockade of single cytokines was also effective, while for the latter the complete cocktail of 3 antibodies was required. The results are quite interesting, and concur with other data suggesting that once type-2 inflammatory responses are established any one of these cytokines can promote continued inflammation. While the data are quite nice, they are also somewhat descriptive. The authors need to provide some data on the identity of the responding cell population in the gut, (DCs, Th2 cells, or ILC2). For example, can transfer of CD4 T cells from sensitized mice into naive mice transfer disease upon challenge?

We now include data (Figure 5) that show that the MCT/EW model of food allergy stimulates large increases in Th2 cells and mast cells and smaller increases in DCs and eosinophils in the lamina propria, but no significant increase in ILC2s, as they are conventionally defined. This is similar to what has been reported for BALB/c food allergy models that use i.p. OVA/alum or epicutaneous OVA/vitamin D for priming, while o.g. priming with cholera toxin does not cause appreciable increases in Th2 cells or ILC2s in the lamina propria (Chen, C. Y., Lee, J. B., Liu, B., Ohta, S., Wang, P. Y., Kartashov, A. V., Mugge, L., Abonia, J. P., Barski, A., Izuhara, K., Rothenberg, M. E., Finkelman, F. D., Hogan, S. P., Wang, Y. H. 2015. Induction of interleukin-9-producing mucosal mast cells promotes susceptibility to IgE-mediated experimental food allergy. *Immunity*. 2015. 43:788-802 – see supplemental figures). We also show now that treatment with an anti-pro-Th2 cytokine cocktail suppresses the food allergy-associated increases in lamina propria Th2 cells, mast cells and eosinophils, but not the increase in DCs. These data suggest that Th2 cells are probably more important than ILC2s (as conventionally defined) as a source of type 2 cytokines in the murine food allergy models. We have not performed cell transfer studies because they would neither address the main points of our paper nor clarify the importance of different cell types in our model. This is because several challenges would likely be required following cell transfer to induce the IgE response that is both necessary and sufficient to mediate a hypothermia response to oral antigen challenge (Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, Zimmermann N, Finkelman FD, Rothenberg ME. 2003. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest*. 112:1666-77; Kucuk, Z. Y., Strait, R., Khodoun, M. V., Mahler, A., Hogan, S. and F. D. Finkelman. 2012. Induction and suppression of allergic diarrhea and systemic anaphylaxis in a

mouse model of food allergy. *J. Allergy Clin. Immunol.* 5:1343-1348) and multiple cell types are likely to be affected during this time period.

We look forward to the re-review of our paper.

Best regards,

Fred Finkelman, M.D.
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Professor of Pediatrics

Prevention of food allergy development and suppression of established food allergy by neutralization of TSLP, IL-25 and IL-33.

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Abbreviations: EW, egg white; FA, food allergy; mAb, i.p., intraperitoneal; monoclonal antibody; MCT, medium chain triglycerides; MMCP1, mouse mast cell protease 1; o. g., oral gavage; OVA, ovalbumin, TSLP, thymic stromal lymphopoietin.

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Abstract:

Background: Food allergy (FA) is an increasing problem that has no approved treatment. The pro-Th2 cytokines, IL-25, IL-33 and TSLP, are associated with FA and monoclonal antibodies (mAbs) to these cytokines are reported to suppress murine FA development.

Objective: Determine whether anti-pro-Th2 cytokine mAbs can block both FA maintenance and induction.

Methods: IgE-mediated FA was induced in BALB/c mice by oral gavage (o.g.) with medium chain triglycerides plus egg white (MCT/EW) and was characterized by increased numbers of lamina propria Th2 cells, mast cells shock, and eosinophils, shock (hypothermia), mast cell degranulation (increased serum MMCP1), increased serum IgG1 anti-EW and IgE, and increased IL-4 and IL-13 secretion following MCT/EW challenge. To suppress FA development, mice were injected with anti-IL-25, IL-33R, and/or TSLP monoclonal antibodies prior to the initial o. g. with MCT/EW; to suppress established FA, treatment with the same mAbs was initiated after FA development.

Results: Injection of a mAb to IL-25, IL-33R, or TSLP strongly inhibited FA development. No single mAb to a pro-Th2 cytokine could suppress established FA and optimal FA suppression required treatment with a cocktail of all three anti-pro-Th2 mAbs. Treatment with the three mAb cocktail during initial MCT/EW immunization induced EW tolerance.

Conclusion: All of the pro-Th2 cytokines are required to induce our model of FA, while any pro-Th2 cytokine can maintain established FA. Pro-Th2 cytokines prevent oral tolerance. Combined treatment with antagonists to all three pro-Th2 cytokines or with an inhibitor of pro-Th2 cytokine production may be able to suppress established human FA.

Introduction:

Food allergy (FA) affects ~8% of children and ~4% of adults in the U.S., where it is responsible for 50,000 ER visits and ~150 deaths per year[1-3]. Furthermore, the incidence of FA has been rapidly increasing in the U.S. and other developed countries[1, 3]. There is no approved therapy for this disorder, other than avoidance of foods that cause allergic symptoms and injection of epinephrine, once symptoms have developed. In common with other allergic disorders, FA is primarily a type 2 cytokine disorder, with IL-4, IL-5, IL-9, and IL-13 having pathogenic roles in mouse models of this disease[4-8]. These cytokines induce FA by promoting IgE production, mastocytosis, eosinophilia, increased smooth muscle contractility, intestinal mastocytosis, and intestinal epithelial permeability[9-14]. Recently, three cytokines, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33, that can be produced by epithelial cells[15, 16], have been shown to act through multiple mechanisms on multiple cell types to promote a type 2 cytokine response[16]; for that reason, we refer to them collectively as "pro-Th2 cytokines." The abilities of the pro-Th2 cytokines to induce production of the Th2 cytokines that are directly responsible for FA led to the hypothesis that the pro-Th2 cytokines might be involved in FA induction and even maintenance. The hypothesis that pro-Th2 cytokines are involved in FA induction is supported by results that have been published during the past few years by several teams of investigators[17-22]; however, no published studies have evaluated whether pro-Th2 cytokines are also important for FA maintenance.

Most of these observations that implicate pro-Th2 cytokines in FA have been made in mouse models of this disease. One important variable in murine FA modeling has been the protocols used to induce disease. In general, these have either primed mice

by inoculating them with food allergens through a non-enteric route (e.g., the skin, lungs, or peritoneum (the latter with alum)) before challenging them orally[23-26], or using a toxin (e.g., cholera toxin or staphylococcal enterotoxin B) as an oral adjuvant to sensitize mice to a co-administered food[27, 28]. Recently, we participated in a study that demonstrated that inoculation of mice with food (peanuts or ovalbumin) along with a common food constituent and additive, medium chain triglycerides (MCT), induces IgE-dependent peanut or ovalbumin FA, respectively, without requiring priming through a non-enteric route or the use of a conventional adjuvant[29]. Studies of the mechanisms involved in FA induction by this protocol demonstrated that MCT ingestion increases intestinal epithelial permeability as well as intestinal epithelial expression of each of the pro-Th2 cytokine genes[29]. This study did not, however, test whether any or all of the pro-Th2 cytokines were required for disease induction or maintenance in this system. We have now used the FA model to test the roles of each pro-Th2 cytokine in disease pathogenesis. Our results indicate that disease induction in this model can be blocked by inhibiting any of the pro-Th2 cytokines, while optimal suppression of established disease requires neutralization of all of these cytokines.

Materials and Methods:

Mice. 7-8 week old BALB/c female mice were purchased from the NCI. Animal work was approved by the Cincinnati Children’s Hospital Research Foundation IACUC.

Reagents. Medium chain triglycerides (MCT) (Nestle Health Science, Switzerland) were purchased at a local pharmacy. Anti-IL-33R mAb (which binds to the long form of ST2, the receptor for IL-33) and anti-IL-25 mAb (clone 2C3, originally produced in the Andrew McKenzie laboratory, Cambridge, UK) were obtained from

Janssen pharmaceuticals. 28F12, a hybridoma that produces anti-TSLP mAb was a gift of Dr. Andrew Farr, University of Washington. Egg white (EW) removed sterilely from organic hen's eggs was dialized against double distilled water and centrifuged for 20 min at 3,900 rcf. The supernatant was concentrated with a stirred ultrafitration cell unit (Millipore, USA) with a 10 kDa Diaflo membrane. Protein concentration was evaluated with a BCA protein assay kit (Pierce, USA) according to the manufacturer's protocol.

Immunofluorescence and flow cytometry: To identify cell types among lamina propria (LP) and mesenteric lymph node (MLN) cells, single cell suspensions prepared from these tissues were first stained with phycoerythrin (PE)-conjugated anti- c-Kit (Biolegend, clone 2B8), PE-Cy7-conjugated anti-FcεRIα (Biolegend, clone MAR-1), allophycocyanin (APC)-conjugated anti-IL17RB, fluorescein isothiocyanate (FITC)-conjugated anti-β7 Integrin (BD Biosciences, clone M293), V500-conjugated anti-CD4 (BD Biosciences, clone RM4-5) and APC-Cy7-conjugated anti-CD3 (Biolegend, clone 145-2C11). Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against lineage (Lin) markers CD8α (Biolegend, clone 53-6.7), B220 (Biolegend, clone RA3-6B2), CD11c (BD Biosciences, clone HL3), and Gr-1 (BD Biosciences, clone RB6-8C5). For identifying dendritic cells, LP cells or MLN cells were first stained with PE-conjugated anti-MHC class II (ebioscience, clone NIMR-4), APC-Cy7-conjugated anti-CD11c (ebioscience, clone NIMR-4), FITC-conjugated anti-CD103 (BD Biosciences, clone M290), Pacific Blue-conjugated anti-CD11b (BD Biosciences, clone M1/70), V500-conjugated anti-Gr-1 (Biolegend, clone RB6-8C5), PE-Cy7 conjugated anti-CD3 (BD Biosciences, clone 145-2C11), APC-conjugated anti-CX3CR1 (R&D Systems) and biotinylated antibodies against lineage markers Ter119 and CD19

(BD Biosciences, clones TER-119 and 1D3 respectively). Subsequently, cells were counterstained with PE-Cy7-labeled streptavidin (BD Biosciences). After staining the cells were analyzed with a FACS Canto II (BD Biosciences). The following cell types were identified by the following markers: MC: Lin$^-$ IL-17RB$^-$ c-kit$^+$ Forward scatter/Side scatter high FcεR1$^+$; ILC2: Lin$^-$ Forward/Side scatter low, FcεR1$^-$ c-kit$^-$ IL-17RB$^+$; Th2: B220$^-$ c-kit$^-$ FcεR1$^-$ CD3$^+$ CD4$^+$ IL17RB$^+$; Eosinophils: Lin$^-$ Gr1$^-$ CD11c$^-$ Forward scatter intermediate Side scatter high CD11b$^+$; DC: Lin$^-$ Gr$^-$ CD11c$^+$ MHC class II$^+$; Basophils: Lin$^-$ IL-17RB$^-$ c-kit$^-$ Forward/Side scatter intermediate FcεR1$^+$. Although we also attempted to distinguish DC subpopulations, the numbers of these cells were too small to reliably determine.

Induction of FA. Mice were inoculated by oral gavage (o. g.) through an 18 gauge needle with a spherical tip with 0.1 ml of MCT on day 0 and day 3, then inoculated o.g. with an emulsion (produced by thorough mixing, followed by brief sonication) of 100 µl of MCT and 100 mg of EW (total volume, 400 µl) as specified in the protocols diagrammed in Figs. 1-5. Mice were fasted for 4 hours before each oral treatment.

Pro-TH2 cytokine blockade. IL-25, IL-33, and TSLP were blocked systemically by intraperitoneal (i.p.) injection of mice with the corresponding mAbs 4 or 12 hours before each MCT or MCT/EW treatment. The quantities of blocking mAbs/week/mouse were based on preliminary studies that identified the doses required to block in vivo function: anti-TSLP, 0.5 mg; anti-IL-33R, 0.1 mg; anti-IL-25, 0.5 mg.

Measurement of IL-4, IL-13, IFN-γ, antigen –specific IgG, IgE, and mouse mast cell protease 1 (MMCP-1). In vivo IL-4 and IFN-γ cytokine secretion were measured by in vivo cytokine capture assay (IVCCA) as previously described[30, 31]. In vivo secretion of

IL-13 was measured by a similar procedure, except that mice were injected with 2 µg of biotin-labeled anti-IL-13 mAb (clone 54D1) and ELISA wells were coated with anti-IL-13 mAb 53F5 (both mAbs were obtained from AbbVie (North Chicago, IL)). EW-specific IgG1 was measured by an ELISA in which ELISA plates (Costar, USA) were coated with EW (10 µg/ml) overnight, then washed and loaded with serial dilutions of mouse sera. After washing, wells were sequentially loaded with 1 µg/ml of biotin-anti-mouse IgG1 (eBioscience, USA) followed by 100 ng/ml of HRP-streptavidin and SuperSignal ELISA substrate, Peroxide and Enhancer solution diluted 20-fold in 20 mM Tris-Saline pH 7.2 (Pierce Biotechnology). Serum levels of MMCP-1 and IgE were measured with the corresponding ELISA kits (eBioscience, USA) according to the manufacturer's protocols.

Anaphylaxis. The severity of anaphylactic shock was assessed by change in rectal temperature measured by digital thermometry[32, 33].

Statistics. Differences in temperature and concentrations of MMCP-1, IL-4, IL-13, IFN-γ, IgE and IgG1 anti-EW Ab were compared using Student's t test (GraphPad Prism 4.0; GraphPad software). A one-tailed test was used to test hypotheses that MCT/EW immunization would increase the parameters studied, that an anti-pro-Th2 cytokine mAb or mAbs would decrease these parameters, and that increasing the number of anti-pro-Th2 cytokine mAbs used would further decrease these parameters. A 2-tailed t test was used to compare cell numbers (Fig. 5). A two way ANOVA with Bonferroni post-test was used to compare temperature curves. A p value < 0.05 was considered significant.

Results

Pro-Th2 cytokine antagonists have a lasting effect on development of food allergy. To determine whether our MCT/ovalbumin model of FA could be inhibited by systemic treatment with a combination of neutralizing monoclonal antibodies (mAbs) to all of the pro-Th2 cytokines, we inoculated BALB/c female mice by o. g. with MCT on days 0 and 3, then o. g. every other day with an MCT/EW emulsion. Mice in one group also received i.p. injections of a combination of anti-IL-25, anti-IL-33R, and anti-TSLP mAbs 12 hours before each o.g. inoculation with MCT or MCT/EW, while mice in the other group were injected i.p. with isotype-matched control mAbs. After 3 weeks, mice that had received isotype control mAbs experienced an ~4°C drop in rectal temperature by 30 min after oral gavage with MCT/EW (which was shown in a separate experiment to be IgE-dependent (Fig. 1S)), while the temperature drop following oral challenge was ~1.2° C in mice that had been treated with the anti-pro-Th2 mAb cocktail (Fig. 1). This suppressive effect reflected a >10-fold decrease in serum levels of MMCP1 (which reflects mucosal mast cell degranulation[32]) and IgG1 anti-EW Ab, as well as an ~3-fold decrease in total serum IgE levels. This suppressive effect was persistent; when these mice were inoculated o.g. with EW/MCT for an additional 5 weeks in the absence of mAb injections the mice that had initially been treated with anti-pro-Th2 mAbs continued to show considerable suppression of development of shock and IgG1, IgE and MMCP1 responses (Figure 1).

IL-25, IL-33 and TSLP are all required for development of FA in EW + MCT-inoculated mice. To determine which of the pro-Th2 cytokines are required for development of FA in our model, mice were not immunized or were inoculated o. g. with MCT, then EW/MCT, as in our initial experiment and were treated i.p. with isotype

control mAbs, anti-TSLP, anti-IL-25, or anti-IL-33R mAb, or a combination of all 3 of these mAbs (Fig. 2A). After 3 weeks of this treatment, shock (>1°C of hypothermia) in response to EW/MCT challenge developed in mice treated with the control mAbs, but not in mice treated with any of the anti-pro-Th2 cytokine mAbs (Fig. 2B). Suppression of development of shock (hypothermia) was complete in mice treated with anti-TSLP mAb, anti-IL-25 mAb or with the mAb cocktail, while a small temperature drop was seen in anti-IL-33R mAb-treated mice. Anti-TSLP mAb suppressed IL-4 and IL-13 responses to basal levels and was more effective than either anti-IL-25 or anti-IL-33R mAb at suppressing the IL-4 and MMCP1 responses (Fig. 2C). Anti-TSLP and anti-IL-33R mAbs were more effective than anti-IL-25 mAb at suppressing IL-13 production. The mAb cocktail was slightly more effective than any of the single mAbs at suppressing the MMCP1 response, but otherwise resembled anti-TSLP mAb in its effects; there was a non-significant trend towards decreased MMCP1 in anti-IL-25 and anti-IL-33 mAb-treated mice. Importantly, the effects of the anti-pro-Th2 cytokines resulted from suppression of the Th2 response without a corresponding shift to a Th1 response, as judged from the lack of a significant increase in IFN-γ secretion in anti-pro-Th2 cytokine mAb-treated mice (Fig. 2C). Serum IgG1 anti-EW and IgE levels were only decreased significantly in mice that had received all 3 anti-pro-Th2 cytokine mAbs; the decreased IgE levels were similar to those in unimmunized mice, but IgG1 anti-EW Ab levels were still increased ~5,000-fold above those in unimmunized mice (Fig. 2C).

Established FA is effectively suppressed by an anti-pro-Th2 mAb cocktail.

Because induction of our model of FA was most effectively suppressed by either anti-TSLP mAb or by a cocktail of all 3 anti-pro-Th2 cytokine mAbs, we evaluated the ability

of each of these mAb treatments to suppress FA that had been established by o. g. inoculation of mice with MCT, then EW/MCT for a total of 4 weeks prior to the initiation of mAb treatment (Fig. 3A). Mice were then inoculated o.g. with MCT/EW for an additional 4 weeks, but also received one of the i.p. mAb treatments. At the end of this 4 week treatment period, the hypothermia response to EW/MCT oral challenge was not affected by anti-TSLP mAb, by itself, but was considerably suppressed by the mAb cocktail (Fig. 3B and D). In the same experiment, the MMCP1 response to MCT/EW challenge was not affected by anti-TSLP mAb alone, but was suppressed by ~80% by the mAb cocktail (Fig. 3C) ; the cocktail was also more effective than anti-TSLP mAb alone at suppressing serum IgE and IgG1 anti-EW Ab levels (Fig. 3B and D). In an additional experiment with mice that were induced to develop FA prior to the initiation of mAb treatment (Figure 4), 24 days of treatment with the mAb cocktail totally suppressed the development of shock and decreased the MMCP1 response to oral challenge by >90%. The same treatment decreased IL-4 and IL-13 responses to oral challenge by 80-90% and total serum IgE and IgG1 anti-EW Ab levels by ~50%. A combination of anti-TSLP and anti-IL-33R mAbs appeared to show less complete ability to suppress FA in this time frame, while combinations of anti-TSLP and anti-IL-25, or anti-IL-25 and anti-IL-33R mAbs were even less effective (Fig. 4).

Maintenance of cellular changes in FA is pro-Th2 cytokine-dependent. To evaluate the cellular changes that accompany the development of FA in our model, we inoculated mice twice a week o.g. for 5 weeks to induce FA (defined as a temperature drop >2°C in response to o.g. challenge), then continued these o.g. inoculations for an additional 5 weeks, but injected mice i.p. with all 3 anti-pro-Th2 cytokine mAbs or

isotype control mAbs 4 h before each o.g. inoculation (Fig. 5A). At the end of this 10 week period, control mAb-treated mice, but not anti-pro-Th2 cytokine mAb-treated continued to develop hypothermia in response to o.g. MCT/EW (not shown). Studies of lamina propria and MLN cells obtained at this time showed large, significant increases in numbers of Th2 cells and mast cells and smaller significant increases in numbers of eosinophils and dendritic cells in the isotype control mAb-treated mice (Fig. 5B). No increases in ILC2 were observed, as compared to untreated mice. Treatment with the cocktail of anti-pro-Th2 cytokine mAbs suppressed the increases in lamina propria Th2 cell, mast cell, and eosinophil number, but not the increase in dendritic cell number. Induction of FA did not significantly increase any of these cell populations in MLN (Fig. 5B).

Discussion:

Our studies with MCT/EW-induced FA have resulted in five important findings that build on our previous observation that MCT induces an intestinal epithelial IL-25, IL-33 and TSLP response[29]: 1) treatment with a blocking mAb to any of these pro-Th2 cytokines inhibits FA development; 2) treatment with a cocktail of all three pro-Th2 cytokine blocking mAbs during oral exposure of immunologically naïve mice to MCT/EW leads to EW tolerance, instead of FA; 3) treatment with all three mAbs is required to optimally suppress established FA; 4) induction of FA in our system is accompanied by increases in lamina propria Th2 cells, mast cells, eosinophils, and dendritic cells, but not ILC2s (as defined in “materials and methods”); and 5) the increases in Th2 cell, mast cell, and eosinophil number are suppressed by anti-pro-Th2 cytokine mAb treatment. Thus, all three pro-Th2 cytokines are required to induce FA in

our model and, once induced, any of these cytokines is sufficient to at least partially maintain this disorder.

Several previous studies have investigated the importance of the pro-Th2 cytokines for FA induction. Studies in which sensitization to peanut or OVA was induced by epicutaneous administration of these antigens, without additional adjuvants, revealed requirements for IL-33 and TSLP in one case, without investigating whether IL-25 was required[18], or TSLP and IL-25 in another case, without investigating whether there was a requirement for IL-33[20]. Additional studies that used a similar approach for sensitization reported a requirement for TSLP, without investigating whether there was also a requirement for IL-25 or IL-33[17]. These results are consistent with the results of our MCT/EW model, in that taken together, they suggest that all three pro-Th2 cytokines are required to induce FA in the absence of additional adjuvants. In contrast, studies that used cholera toxin as an adjuvant for FA induction reported a requirement for IL-33, but not for TSLP[19]; this suggests that at least some of the adjuvants that have commonly been used in murine FA models can bypass the requirement for the latter cytokine.

Although several studies have, thus, examined pro-Th2 cytokine requirements for FA induction, ours, to the best of our knowledge, is the first to evaluate the clinically relevant question of whether pro-Th2 cytokines are required to maintain established FA. Because pro-Th2 cytokines are known to be important in the induction of type 2 cytokine production by Th2 cells[16] and established Th2 cells can lose their ability to switch to production of other cytokines[34], it was possible that the pro-Th2 cytokines had little importance in maintaining type 2 cytokine-dependent FA. Indeed, had we only neutralized one pro-Th2 cytokine at a time, we might have reached that conclusion,

because neither anti-TSLP mAb alone (Fig. 3) nor the combination of anti-IL-25 and anti-IL-33 mAbs (Fig. 4) had a significant effect on established FA; even the combination of anti-IL-33 and TSLP mAbs had only a moderate suppressive effect. Only the combination of mAbs to all three pro-Th2 cytokines was able to strongly suppress established FA within 3-4 weeks (Fig. 4). This suggests that once established, any of the pro-Th2 cytokines can maintain FA, at least to some extent. It is not yet known whether the pro-Th2 cytokine contribution to FA maintenance reflects a need for these cytokines to maintain type 2 cytokine production by Th2 cells. If so, this would suggest that Th2 commitment is not irreversible in vivo or that persistent cytokine production and/or survival of this population, or its replacement with fresh Th2 cells, requires continuing pro-Th2 cytokine stimulation. Alternatively, continuing pro-Th2 cytokine stimulation of type 2 cytokine production by type 2 innate lymphoid cells, basophils and/or mast cells may be required to maintain a sufficient type 2 cytokine response to permit FA persistence. In either case, to the extent that our results reflect the pathophysiology of human FA, they suggest that successful therapy would require inhibition of the pro-Th2 cytokine triad or suppression of the production of all three of these cytokines, perhaps through an effect on intestinal epithelial cells. It is possible, however, that studies with different animal models of FA would show different pro-Th2 cytokine dependence for FA maintenance.

Our observations also demonstrate the cellular changes that accompany the development of our model of food allergy and the pro-Th2 cytokine requirement for maintenance of these changes. After 10 weeks of MCT/EW inoculation, we saw large increases in Th2 and mast cell number and smaller increases in eosinophil and dendritic

cell number in the lamina propria of treated mice, without any increase in lamina propria ILC2s or basophils or an increase in any cell type in mesenteric lymph node. These changes are similar to those observed in food allergy models that relied on i.p. inoculation with alum or epicutaneous immunization to prime for food allergy development.[12] More importantly, our data demonstrate that 5 weeks of treatment with mAbs that block all 3 pro-Th2 cytokines prevents or, more likely, reverses the increases in lamina propria Th2 cells, mast cells and eosinophils, but not dendritic cells. The lack of association of food allergy models with an increase in lamina propria ILC2s is surprising in view of the potent pro-Th2 cytokine effects on ILC2 survival and proliferation[15] and the considerable increase in ILC2s in intestinal worm infection models.[35,-39] Our results suggest that one or more additional stimulus that is induced by worm infection, but not by the FA models, is required for proliferation of these cells. They also suggest that cytokine production by Th2 cells and mast cells may be the predominant source of Th2 cytokines in the FA models, although it is also possible that the pro-Th2 cytokines produced in these models increase ILC2 cytokine production without driving their proliferation or that FA is accompanied by an increase in an ILC2 subpopulation that was not detected by our markers.

The relevance of our observations to human FA is also uncertain; few studies of pro-Th2 cytokines have been performed in human food-allergic individuals, although, TSLP and IL-33 have been associated with human eosinophilic esophagitis[40, 41] and all three of the pro-Th2 cytokines have been associated with human atopic dermatitis[42-44], a FA-associated skin disorder[17, 45, 46]. We are aware of only two clinical trials of antibodies to pro-Th2 cytokines in human allergic disorders: anti-TSLP mAb inhibited responses to

allergen challenge in a small trial in patients with mild asthma[47], while an anti-IL-17RA mAb, which was designed to inhibit responses to IL-25 and other IL-17 family cytokines, had no significant effect in patients with moderate to severe asthma[48]. No clinical trials with agents designed to suppress more than one pro-Th2 cytokine have been reported in PubMed or ClinicalTrial.gov; the results of our study suggest that such human trials might be required for clinically relevant suppression of established FA.

Acknowledgements: This work was supported by a Merit Award (FDF and MVK) from the U.S. Department of Veterans Affairs, a grant (FDF and MVK) from Food Allergy Research and Education (FARE), NIH grants R01 AI112626-01 (Y-HW and FDF) and 2U19 AI070235-11 (Y-HW), and Department of Defense grant W81XWH-15-1-0517 (Y-HW and FDF). We thank Dr. Andrew Farr for his gift of an anti-mouse TSLP mAb-secreting hybridoma, Janssen Pharmaceuticals for providing purified mAbs to mouse IL-25 and mouse IL-33 and AbbVie for providing purified antibodies to mouse IL-13.

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Figure legends

Figure 1. Pro-Th2 cytokine antagonists have a lasting effect on development of food allergy. A. BALB/c female mice, 4-6 mice per group, were inoculated o. g. with 100 µl of MCT on days 0 and 3, then inoculated o.g. with MCT/EW emulsion every other day for 3 weeks. One group was injected i.p. with a cocktail of anti-TSLP/anti-IL-33R/anti-IL-25 mAbs 12 hours before each MCT/EW dose, while the other group was injected i.p. with isotype control mAbs. Rectal temperatures were determined for the hour after the last o.g. inoculation (B, left panel) and mice were bled 4 hr after this inoculation. Treatment with anti-pro-Th2 cytokine mAbs and isotype control mAbs was then discontinued, but all mice were inoculated o. g. every other day for an additional 5 weeks with MCT/EW. Mice were again followed for decreases in rectal temperature for 1 hour after the last o.g. inoculation (B, right panel). Mice were again bled 4 hours after this o. g. inoculation and total IgE, EW-specific IgG1, and MMCP1 levels were evaluated by ELISA (C). For all figures, * indicates $p<0.05$, as compared to isotype control treated mice.

Figure 2. IL-25, IL-33, and TSLP are all required for development of food allergy in EW + MCT-inoculated mice. A. BALB/c mice, 4-6/group, were fasted for 4 hours and left untreated or inoculated o. g. with 100 µl of MCT on day 0 and day 3. MCT-treated mice were then inoculated o.g. with MCT/EW emulsion every other day for three weeks. Mice were also injected i.p. 12 h before each MCT/EW inoculation with anti-TSLP mAb, anti-IL-25 mAb, anti-IL-33R mAb, a cocktail of anti-TSLP/anti-IL-33/anti-IL-25 mAbs, or with isotype control mAbs 12 h before each MCT/EW dose. Rectal temperatures were determined for the hour after the last o. g. inoculation (B). Mice were bled 4 hours after

this inoculation. IL-4, IL-13, and IFN-γ secretion were evaluated by IVCCA; while serum levels of MMCP1, IgE and IgG1 anti-EW were determined by ELISA (C). In this and other figures, brackets with asterisks indicate a statistically significant ($p<0.05$) different between the groups connected by the bracket.

Figure 3. Established food allergy is suppressed by an anti-pro-Th2 mAb cocktail. A. BALB/c mice were fasted for 4 hours and sensitized with two oral doses of MCT on day 0 and day 3. Then mice were treated with MCT/EW emulsion every other day for four weeks. Mice that developed > 4° C maximum temperature drop were divided into 3 groups of 5 mice per group. All groups were inoculated o. g. with MCT/EW emulsion twice a week for 4 more weeks. The different groups were also injected i.p. with anti-TSLP mAb, with the cocktail of anti-TSLP/anti-IL-33R/anti-IL-25 mAbs, or with isotype control mAbs 12 h before each MCT/EW inoculation. Decreases in rectal temperature were determined for the hour after the last MCT/EW inoculation (B and D). Mice were bled 4 hours after the last o. g. inoculation for determination of serum MMCP1 levels (C) as well as serum IgE levels and IgG1 anti-EW titers (D).

Figure 4. Combined pro-Th2 cytokine blockade is required for effective suppression of established food allergy. A. BALB/c mice were fasted for 4 hours, then inoculated o.g. with 100 µl of MCT on day 0 and day 3. Mice were then kept unimmunized or were inoculated o. g. with MCT/EW emulsion twice a week for four weeks. Mice that developed significant shock (>4° C maximum temperature drop) were divided into 5 groups of 5 mice/group. All groups were then inoculated o. g. with MCT/EW emulsion twice a week for an additional 24 days. Different groups of MCT/EW-immune mice were injected i.p. with the following mAb combinations 12 hours before each o. g. inoculation

with MCT + EW: anti-TSLP + anti-IL-33R mAb; anti-TSLP + anti-IL-25 mAb, anti-IL-25 + anti-IL-33R mAb, anti-TSLP + anti-IL-33R + anti-IL-25 mAb, or isotype control mAbs. Maximal decreases in rectal temperature were determined for the hour following the o.g. inoculation just prior to the initiation of mAb treatment (B, day 0) and for the hour following the o.g. inoculations after 14 and 24 days of mAb treatment (B). Mice were bled 4 h after the day 24 o.g. inoculation to determine levels of IL-4 and IL-13 secretion, MMCP1 response and serum IgE and IgG1 anti-EW levels (C).

Figure 5. Maintenance of increased lamina propria Th2 cell, mast cell (MC), and eosinophil numbers in food allergy is pro-Th2 cytokine-dependent. A. BALB/c mice (4/group) were left untreated (naïve) or were inoculated o.g. with MCT for 3 day, followed by MCT/EW every 4 days for 5 weeks. Following this, mice that had developed a temperature drop of at least 2°C following o.g. inoculation continued to receive o.g. MCT/EW every 4 days for an additional 5 weeks; half of these mice were injected i.p. with anti-TSLP/IL-25/IL-33 mAbs, half with isotype control mAbs, 4 h before each o.g. inoculation. Following the last o.g. inoculation, lamina propria (LP) and mesenteric lymph node (MLN) single cell suspensions were prepared, stained for Th2 cell, ILC2, mast cell, basophil, eosinophil or dendritic cell markers and analyzed for number of each cell type (B) by Coulter counting and flow cytometry.

Supplemental Figure.

Figure S1. Development of hypothermia in response to ingested Ag is IgE-dependent in food-allergic mice. BALB/c mice (4/group) were inoculated o.g. with MCT for 3 days, then with MCT/EW twice a week for 5 weeks, until they developed hypothermia in

response to o.g. inoculation. Mice were then injected i.p. with 500 µg of anti-IgE mAb (EM-95), 500 µg of anti-FcγRIIB/RIII mAb (2.4G2), both mAbs, or isotype control mAbs. One day later, mice were challenged o.g. with MCT/EW and rectal temperatures were followed for the next 60 minutes.

*Revision - Unmarked Manuscript

Prevention of food allergy development and suppression of established food allergy by neutralization of TSLP, IL-25 and IL-33.

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Abbreviations: EW, egg white; FA, food allergy; mAb, i.p., intraperitoneal; monoclonal antibody; MCT, medium chain triglycerides; MMCP1, mouse mast cell protease 1; o. g., oral gavage; OVA, ovalbumin, TSLP, thymic stromal lymphopoietin.

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Abstract:

Background: Food allergy (FA) is an increasing problem that has no approved treatment. The pro-Th2 cytokines, IL-25, IL-33 and TSLP, are associated with FA and monoclonal antibodies (mAbs) to these cytokines are reported to suppress murine FA development.

Objective: Determine whether anti-pro-Th2 cytokine mAbs can block both FA maintenance and induction.

Methods: IgE-mediated FA was induced in BALB/c mice by oral gavage (o.g.) with medium chain triglycerides plus egg white (MCT/EW) and was characterized by increased numbers of lamina propria Th2 cells, mast cells shock, and eosinophils, shock (hypothermia), mast cell degranulation (increased serum MMCP1), increased serum IgG1 anti-EW and IgE, and increased IL-4 and IL-13 secretion following MCT/EW challenge. To suppress FA development, mice were injected with anti-IL-25, IL-33R, and/or TSLP monoclonal antibodies prior to the initial o. g. with MCT/EW; to suppress established FA, treatment with the same mAbs was initiated after FA development.

Results: Injection of a mAb to IL-25, IL-33R, or TSLP strongly inhibited FA development. No single mAb to a pro-Th2 cytokine could suppress established FA and optimal FA suppression required treatment with a cocktail of all three anti-pro-Th2 mAbs. Treatment with the three mAb cocktail during initial MCT/EW immunization induced EW tolerance.

Conclusion: All of the pro-Th2 cytokines are required to induce our model of FA, while any pro-Th2 cytokine can maintain established FA. Pro-Th2 cytokines prevent oral tolerance. Combined treatment with antagonists to all three pro-Th2 cytokines or with an inhibitor of pro-Th2 cytokine production may be able to suppress established human FA.

Introduction:

Food allergy (FA) affects ~8% of children and ~4% of adults in the U.S., where it is responsible for 50,000 ER visits and ~150 deaths per year[1-3]. Furthermore, the incidence of FA has been rapidly increasing in the U.S. and other developed countries[1, 3]. There is no approved therapy for this disorder, other than avoidance of foods that cause allergic symptoms and injection of epinephrine, once symptoms have developed. In common with other allergic disorders, FA is primarily a type 2 cytokine disorder, with IL-4, IL-5, IL-9, and IL-13 having pathogenic roles in mouse models of this disease[4-8]. These cytokines induce FA by promoting IgE production, mastocytosis, eosinophilia, increased smooth muscle contractility, intestinal mastocytosis, and intestinal epithelial permeability[9-14]. Recently, three cytokines, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33, that can be produced by epithelial cells[15, 16], have been shown to act through multiple mechanisms on multiple cell types to promote a type 2 cytokine response[16]; for that reason, we refer to them collectively as “pro-Th2 cytokines.” The abilities of the pro-Th2 cytokines to induce production of the Th2 cytokines that are directly responsible for FA led to the hypothesis that the pro-Th2 cytokines might be involved in FA induction and even maintenance. The hypothesis that pro-Th2 cytokines are involved in FA induction is supported by results that have been published during the past few years by several teams of investigators[17-22]; however, no published studies have evaluated whether pro-Th2 cytokines are also important for FA maintenance.

Most of these observations that implicate pro-Th2 cytokines in FA have been made in mouse models of this disease. One important variable in murine FA modeling has been the protocols used to induce disease. In general, these have either primed mice

by inoculating them with food allergens through a non-enteric route (e.g., the skin, lungs, or peritoneum (the latter with alum)) before challenging them orally[23-26], or using a toxin (e.g., cholera toxin or staphylococcal enterotoxin B) as an oral adjuvant to sensitize mice to a co-administered food[27, 28]. Recently, we participated in a study that demonstrated that inoculation of mice with food (peanuts or ovalbumin) along with a common food constituent and additive, medium chain triglycerides (MCT), induces IgE-dependent peanut or ovalbumin FA, respectively, without requiring priming through a non-enteric route or the use of a conventional adjuvant[29]. Studies of the mechanisms involved in FA induction by this protocol demonstrated that MCT ingestion increases intestinal epithelial permeability as well as intestinal epithelial expression of each of the pro-Th2 cytokine genes[29]. This study did not, however, test whether any or all of the pro-Th2 cytokines were required for disease induction or maintenance in this system. We have now used the FA model to test the roles of each pro-Th2 cytokine in disease pathogenesis. Our results indicate that disease induction in this model can be blocked by inhibiting any of the pro-Th2 cytokines, while optimal suppression of established disease requires neutralization of all of these cytokines.

Materials and Methods:

Mice. 7-8 week old BALB/c female mice were purchased from the NCI. Animal work was approved by the Cincinnati Children's Hospital Research Foundation IACUC.

Reagents. Medium chain triglycerides (MCT) (Nestle Health Science, Switzerland) were purchased at a local pharmacy. Anti-IL-33R mAb (which binds to the long form of ST2, the receptor for IL-33) and anti-IL-25 mAb (clone 2C3, originally produced in the Andrew McKenzie laboratory, Cambridge, UK) were obtained from

Janssen pharmaceuticals. 28F12, a hybridoma that produces anti-TSLP mAb was a gift of Dr. Andrew Farr, University of Washington. Egg white (EW) removed sterilely from organic hen's eggs was dialized against double distilled water and centrifuged for 20 min at 3,900 rcf. The supernatant was concentrated with a stirred ultrafitration cell unit (Millipore, USA) with a 10 kDa Diaflo membrane. Protein concentration was evaluated with a BCA protein assay kit (Pierce, USA) according to the manufacturer's protocol.

Immunofluorescence and flow cytometry: To identify cell types among lamina propria (LP) and mesenteric lymph node (MLN) cells, single cell suspensions prepared from these tissues were first stained with phycoerythrin (PE)-conjugated anti- c-Kit (Biolegend, clone 2B8), PE-Cy7-conjugated anti-FcεRIα (Biolegend, clone MAR-1), allophycocyanin (APC)-conjugated anti-IL17RB, fluorescein isothiocyanate (FITC)-conjugated anti-β7 Integrin (BD Biosciences, clone M293), V500-conjugated anti-CD4 (BD Biosciences, clone RM4-5) and APC-Cy7-conjugated anti-CD3 (Biolegend, clone 145-2C11). Subsequently, cells were counterstained with PerCP-Cy5.5-conjugated monoclonal antibodies against lineage (Lin) markers CD8α (Biolegend, clone 53-6.7), B220 (Biolegend, clone RA3-6B2), CD11c (BD Biosciences, clone HL3), and Gr-1 (BD Biosciences, clone RB6-8C5). For identifying dendritic cells, LP cells or MLN cells were first stained with PE-conjugated anti-MHC class II (ebioscience, clone NIMR-4), APC-Cy7-conjugated anti-CD11c (ebioscience, clone NIMR-4), FITC-conjugated anti-CD103 (BD Biosciences, clone M290), Pacific Blue-conjugated anti-CD11b (BD Biosciences, clone M1/70), V500-conjugated anti-Gr-1 (Biolegend, clone RB6-8C5), PE-Cy7 conjugated anti-CD3 (BD Biosciences, clone 145-2C11), APC-conjugated anti-CX3CR1 (R&D Systems) and biotinylated antibodies against lineage markers Ter119 and CD19

(BD Biosciences, clones TER-119 and 1D3 respectively). Subsequently, cells were counterstained with PE-Cy7-labeled streptavidin (BD Biosciences). After staining the cells were analyzed with a FACS Canto II (BD Biosciences). The following cell types were identified by the following markers: MC: Lin^{-} IL-17RB^{-} c-kit^{+} Forward scatter/Side scatter high FcεR1^{+}; ILC2: Lin^{-} Forward/Side scatter low, FcεR1^{-} c-kit^{-} IL-17RB^{+}; Th2: B220^{-} c-kit^{-} FcεR1^{-} CD3^{+} CD4^{+} IL17RB^{+}; Eosinophils: Lin^{-} Gr1^{-} CD11c^{-} Forward scatter intermediate Side scatter high CD11b^{+}; DC: Lin^{-} Gr^{-} CD11c^{+} MHC class II^{+}; Basophils: Lin^{-} IL-17RB^{-} c-kit^{-} Forward/Side scatter intermediate FcεR1^{+}. Although we also attempted to distinguish DC subpopulations, the numbers of these cells were too small to reliably determine.

Induction of FA. Mice were inoculated by oral gavage (o. g.) through an 18 gauge needle with a spherical tip with 0.1 ml of MCT on day 0 and day 3, then inoculated o.g. with an emulsion (produced by thorough mixing, followed by brief sonication) of 100 µl of MCT and 100 mg of EW (total volume, 400 µl) as specified in the protocols diagrammed in Figs. 1-5. Mice were fasted for 4 hours before each oral treatment.

Pro-TH2 cytokine blockade. IL-25, IL-33, and TSLP were blocked systemically by intraperitoneal (i.p.) injection of mice with the corresponding mAbs 4 or 12 hours before each MCT or MCT/EW treatment. The quantities of blocking mAbs/week/mouse were based on preliminary studies that identified the doses required to block in vivo function: anti-TSLP, 0.5 mg; anti-IL-33R, 0.1 mg; anti-IL-25, 0.5 mg.

Measurement of IL-4, IL-13, IFN-γ, antigen –specific IgG, IgE, and mouse mast cell protease 1 (MMCP-1). In vivo IL-4 and IFN-γ cytokine secretion were measured by in vivo cytokine capture assay (IVCCA) as previously described[30, 31]. In vivo secretion of

IL-13 was measured by a similar procedure, except that mice were injected with 2 µg of biotin-labeled anti-IL-13 mAb (clone 54D1) and ELISA wells were coated with anti-IL-13 mAb 53F5 (both mAbs were obtained from AbbVie (North Chicago, IL)). EW-specific IgG1 was measured by an ELISA in which ELISA plates (Costar, USA) were coated with EW (10 µg/ml) overnight, then washed and loaded with serial dilutions of mouse sera. After washing, wells were sequentially loaded with 1 µg/ml of biotin-anti-mouse IgG1 (eBioscience, USA) followed by 100 ng/ml of HRP-streptavidin and SuperSignal ELISA substrate, Peroxide and Enhancer solution diluted 20-fold in 20 mM Tris-Saline pH 7.2 (Pierce Biotechnology). Serum levels of MMCP-1 and IgE were measured with the corresponding ELISA kits (eBioscience, USA) according to the manufacturer's protocols.

Anaphylaxis. The severity of anaphylactic shock was assessed by change in rectal temperature measured by digital thermometry[32, 33].

Statistics. Differences in temperature and concentrations of MMCP-1, IL-4, IL-13, IFN-γ, IgE and IgG1 anti-EW Ab were compared using Student's t test (GraphPad Prism 4.0; GraphPad software). A one-tailed test was used to test hypotheses that MCT/EW immunization would increase the parameters studied, that an anti-pro-Th2 cytokine mAb or mAbs would decrease these parameters, and that increasing the number of anti-pro-Th2 cytokine mAbs used would further decrease these parameters. A 2-tailed t test was used to compare cell numbers (Fig. 5). A two way ANOVA with Bonferroni post-test was used to compare temperature curves. A p value < 0.05 was considered significant.

Results

Pro-Th2 cytokine antagonists have a lasting effect on development of food allergy. To determine whether our MCT/ovalbumin model of FA could be inhibited by systemic treatment with a combination of neutralizing monoclonal antibodies (mAbs) to all of the pro-Th2 cytokines, we inoculated BALB/c female mice by o. g. with MCT on days 0 and 3, then o. g. every other day with an MCT/EW emulsion. Mice in one group also received i.p. injections of a combination of anti-IL-25, anti-IL-33R, and anti-TSLP mAbs 12 hours before each o.g. inoculation with MCT or MCT/EW, while mice in the other group were injected i.p. with isotype-matched control mAbs. After 3 weeks, mice that had received isotype control mAbs experienced an ~4°C drop in rectal temperature by 30 min after oral gavage with MCT/EW (which was shown in a separate experiment to be IgE-dependent (Fig. 1S)), while the temperature drop following oral challenge was ~1.2° C in mice that had been treated with the anti-pro-Th2 mAb cocktail (Fig. 1). This suppressive effect reflected a >10-fold decrease in serum levels of MMCP1 (which reflects mucosal mast cell degranulation[32]) and IgG1 anti-EW Ab, as well as an ~3-fold decrease in total serum IgE levels. This suppressive effect was persistent; when these mice were inoculated o.g. with EW/MCT for an additional 5 weeks in the absence of mAb injections the mice that had initially been treated with anti-pro-Th2 mAbs continued to show considerable suppression of development of shock and IgG1, IgE and MMCP1 responses (Figure 1).

IL-25, IL-33 and TSLP are all required for development of FA in EW + MCT-inoculated mice. To determine which of the pro-Th2 cytokines are required for development of FA in our model, mice were not immunized or were inoculated o. g. with MCT, then EW/MCT, as in our initial experiment and were treated i.p. with isotype

control mAbs, anti-TSLP, anti-IL-25, or anti-IL-33R mAb, or a combination of all 3 of these mAbs (Fig. 2A). After 3 weeks of this treatment, shock (>1°C of hypothermia) in response to EW/MCT challenge developed in mice treated with the control mAbs, but not in mice treated with any of the anti-pro-Th2 cytokine mAbs (Fig. 2B). Suppression of development of shock (hypothermia) was complete in mice treated with anti-TSLP mAb, anti-IL-25 mAb or with the mAb cocktail, while a small temperature drop was seen in anti-IL-33R mAb-treated mice. Anti-TSLP mAb suppressed IL-4 and IL-13 responses to basal levels and was more effective than either anti-IL-25 or anti-IL-33R mAb at suppressing the IL-4 and MMCP1 responses (Fig. 2C). Anti-TSLP and anti-IL-33R mAbs were more effective than anti-IL-25 mAb at suppressing IL-13 production. The mAb cocktail was slightly more effective than any of the single mAbs at suppressing the MMCP1 response, but otherwise resembled anti-TSLP mAb in its effects; there was a non-significant trend towards decreased MMCP1 in anti-IL-25 and anti-IL-33 mAb-treated mice. Importantly, the effects of the anti-pro-Th2 cytokines resulted from suppression of the Th2 response without a corresponding shift to a Th1 response, as judged from the lack of a significant increase in IFN-γ secretion in anti-pro-Th2 cytokine mAb-treated mice (Fig. 2C). Serum IgG1 anti-EW and IgE levels were only decreased significantly in mice that had received all 3 anti-pro-Th2 cytokine mAbs; the decreased IgE levels were similar to those in unimmunized mice, but IgG1 anti-EW Ab levels were still increased ~5,000-fold above those in unimmunized mice (Fig. 2C).

Established FA is effectively suppressed by an anti-pro-Th2 mAb cocktail.

Because induction of our model of FA was most effectively suppressed by either anti-TSLP mAb or by a cocktail of all 3 anti-pro-Th2 cytokine mAbs, we evaluated the ability

of each of these mAb treatments to suppress FA that had been established by o. g. inoculation of mice with MCT, then EW/MCT for a total of 4 weeks prior to the initiation of mAb treatment (Fig. 3A). Mice were then inoculated o.g. with MCT/EW for an additional 4 weeks, but also received one of the i.p. mAb treatments. At the end of this 4 week treatment period, the hypothermia response to EW/MCT oral challenge was not affected by anti-TSLP mAb, by itself, but was considerably suppressed by the mAb cocktail (Fig. 3B and D). In the same experiment, the MMCP1 response to MCT/EW challenge was not affected by anti-TSLP mAb alone, but was suppressed by ~80% by the mAb cocktail (Fig. 3C) ; the cocktail was also more effective than anti-TSLP mAb alone at suppressing serum IgE and IgG1 anti-EW Ab levels (Fig. 3B and D). In an additional experiment with mice that were induced to develop FA prior to the initiation of mAb treatment (Figure 4), 24 days of treatment with the mAb cocktail totally suppressed the development of shock and decreased the MMCP1 response to oral challenge by >90%. The same treatment decreased IL-4 and IL-13 responses to oral challenge by 80-90% and total serum IgE and IgG1 anti-EW Ab levels by ~50%. A combination of anti-TSLP and anti-IL-33R mAbs appeared to show less complete ability to suppress FA in this time frame, while combinations of anti-TSLP and anti-IL-25, or anti-IL-25 and anti-IL-33R mAbs were even less effective (Fig. 4).

Maintenance of cellular changes in FA is pro-Th2 cytokine-dependent. To evaluate the cellular changes that accompany the development of FA in our model, we inoculated mice twice a week o.g. for 5 weeks to induce FA (defined as a temperature drop >2°C in response to o.g. challenge), then continued these o.g. inoculations for an additional 5 weeks, but injected mice i.p. with all 3 anti-pro-Th2 cytokine mAbs or

isotype control mAbs 4 h before each o.g. inoculation (Fig. 5A). At the end of this 10 week period, control mAb-treated mice, but not anti-pro-Th2 cytokine mAb-treated continued to develop hypothermia in response to o.g. MCT/EW (not shown). Studies of lamina propria and MLN cells obtained at this time showed large, significant increases in numbers of Th2 cells and mast cells and smaller significant increases in numbers of eosinophils and dendritic cells in the isotype control mAb-treated mice (Fig. 5B). No increases in ILC2 were observed, as compared to untreated mice. Treatment with the cocktail of anti-pro-Th2 cytokine mAbs suppressed the increases in lamina propria Th2 cell, mast cell, and eosinophil number, but not the increase in dendritic cell number. Induction of FA did not significantly increase any of these cell populations in MLN (Fig. 5B).

Discussion:

Our studies with MCT/EW-induced FA have resulted in five important findings that build on our previous observation that MCT induces an intestinal epithelial IL-25, IL-33 and TSLP response[29]: 1) treatment with a blocking mAb to any of these pro-Th2 cytokines inhibits FA development; 2) treatment with a cocktail of all three pro-Th2 cytokine blocking mAbs during oral exposure of immunologically naïve mice to MCT/EW leads to EW tolerance, instead of FA; 3) treatment with all three mAbs is required to optimally suppress established FA; 4) induction of FA in our system is accompanied by increases in lamina propria Th2 cells, mast cells, eosinophils, and dendritic cells, but not ILC2s (as defined in “materials and methods”); and 5) the increases in Th2 cell, mast cell, and eosinophil number are suppressed by anti-pro-Th2 cytokine mAb treatment. Thus, all three pro-Th2 cytokines are required to induce FA in

our model and, once induced, any of these cytokines is sufficient to at least partially maintain this disorder.

Several previous studies have investigated the importance of the pro-Th2 cytokines for FA induction. Studies in which sensitization to peanut or OVA was induced by epicutaneous administration of these antigens, without additional adjuvants, revealed requirements for IL-33 and TSLP in one case, without investigating whether IL-25 was required[18], or TSLP and IL-25 in another case, without investigating whether there was a requirement for IL-33[20]. Additional studies that used a similar approach for sensitization reported a requirement for TSLP, without investigating whether there was also a requirement for IL-25 or IL-33[17]. These results are consistent with the results of our MCT/EW model, in that taken together, they suggest that all three pro-Th2 cytokines are required to induce FA in the absence of additional adjuvants. In contrast, studies that used cholera toxin as an adjuvant for FA induction reported a requirement for IL-33, but not for TSLP[19]; this suggests that at least some of the adjuvants that have commonly been used in murine FA models can bypass the requirement for the latter cytokine.

Although several studies have, thus, examined pro-Th2 cytokine requirements for FA induction, ours, to the best of our knowledge, is the first to evaluate the clinically relevant question of whether pro-Th2 cytokines are required to maintain established FA. Because pro-Th2 cytokines are known to be important in the induction of type 2 cytokine production by Th2 cells[16] and established Th2 cells can lose their ability to switch to production of other cytokines[34], it was possible that the pro-Th2 cytokines had little importance in maintaining type 2 cytokine-dependent FA. Indeed, had we only neutralized one pro-Th2 cytokine at a time, we might have reached that conclusion,

because neither anti-TSLP mAb alone (Fig. 3) nor the combination of anti-IL-25 and anti-IL-33 mAbs (Fig. 4) had a significant effect on established FA; even the combination of anti-IL-33 and TSLP mAbs had only a moderate suppressive effect. Only the combination of mAbs to all three pro-Th2 cytokines was able to strongly suppress established FA within 3-4 weeks (Fig. 4). This suggests that once established, any of the pro-Th2 cytokines can maintain FA, at least to some extent. It is not yet known whether the pro-Th2 cytokine contribution to FA maintenance reflects a need for these cytokines to maintain type 2 cytokine production by Th2 cells. If so, this would suggest that Th2 commitment is not irreversible in vivo or that persistent cytokine production and/or survival of this population, or its replacement with fresh Th2 cells, requires continuing pro-Th2 cytokine stimulation. Alternatively, continuing pro-Th2 cytokine stimulation of type 2 cytokine production by type 2 innate lymphoid cells, basophils and/or mast cells may be required to maintain a sufficient type 2 cytokine response to permit FA persistence. In either case, to the extent that our results reflect the pathophysiology of human FA, they suggest that successful therapy would require inhibition of the pro-Th2 cytokine triad or suppression of the production of all three of these cytokines, perhaps through an effect on intestinal epithelial cells. It is possible, however, that studies with different animal models of FA would show different pro-Th2 cytokine dependence for FA maintenance.

Our observations also demonstrate the cellular changes that accompany the development of our model of food allergy and the pro-Th2 cytokine requirement for maintenance of these changes. After 10 weeks of MCT/EW inoculation, we saw large increases in Th2 and mast cell number and smaller increases in eosinophil and dendritic

cell number in the lamina propria of treated mice, without any increase in lamina propria ILC2s or basophils or an increase in any cell type in mesenteric lymph node. These changes are similar to those observed in food allergy models that relied on i.p. inoculation with alum or epicutaneous immunization to prime for food allergy development.[12] More importantly, our data demonstrate that 5 weeks of treatment with mAbs that block all 3 pro-Th2 cytokines prevents or, more likely, reverses the increases in lamina propria Th2 cells, mast cells and eosinophils, but not dendritic cells. The lack of association of food allergy models with an increase in lamina propria ILC2s is surprising in view of the potent pro-Th2 cytokine effects on ILC2 survival and proliferation[15] and the considerable increase in ILC2s in intestinal worm infection models.[35,-39] Our results suggest that one or more additional stimulus that is induced by worm infection, but not by the FA models, is required for proliferation of these cells. They also suggest that cytokine production by Th2 cells and mast cells may be the predominant source of Th2 cytokines in the FA models, although it is also possible that the pro-Th2 cytokines produced in these models increase ILC2 cytokine production without driving their proliferation or that FA is accompanied by an increase in an ILC2 subpopulation that was not detected by our markers.

The relevance of our observations to human FA is also uncertain; few studies of pro-Th2 cytokines have been performed in human food-allergic individuals, although, TSLP and IL-33 have been associated with human eosinophilic esophagitis[40, 41] and all three of the pro-Th2 cytokines have been associated with human atopic dermatitis[42-44], a FA-associated skin disorder[17, 45, 46]. We are aware of only two clinical trials of antibodies to pro-Th2 cytokines in human allergic disorders: anti-TSLP mAb inhibited responses to

allergen challenge in a small trial in patients with mild asthma[47], while an anti-IL-17RA mAb, which was designed to inhibit responses to IL-25 and other IL-17 family cytokines, had no significant effect in patients with moderate to severe asthma[48]. No clinical trials with agents designed to suppress more than one pro-Th2 cytokine have been reported in PubMed or ClinicalTrial.gov; the results of our study suggest that such human trials might be required for clinically relevant suppression of established FA.

Acknowledgements: This work was supported by a Merit Award (FDF and MVK) from the U.S. Department of Veterans Affairs, a grant (FDF and MVK) from Food Allergy Research and Education (FARE), NIH grants R01 AI112626-01 (Y-HW and FDF) and 2U19 AI070235-11 (Y-HW), and Department of Defense grant W81XWH-15-1-0517 (Y-HW and FDF). We thank Dr. Andrew Farr for his gift of an anti-mouse TSLP mAb-secreting hybridoma, Janssen Pharmaceuticals for providing purified mAbs to mouse IL-25 and mouse IL-33 and AbbVie for providing purified antibodies to mouse IL-13.

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Figure legends

Figure 1. Pro-Th2 cytokine antagonists have a lasting effect on development of food allergy. A. BALB/c female mice, 4-6 mice per group, were inoculated o. g. with 100 µl of MCT on days 0 and 3, then inoculated o.g. with MCT/EW emulsion every other day for 3 weeks. One group was injected i.p. with a cocktail of anti-TSLP/anti-IL-33R/anti-IL-25 mAbs 12 hours before each MCT/EW dose, while the other group was injected i.p. with isotype control mAbs. Rectal temperatures were determined for the hour after the last o.g. inoculation (B, left panel) and mice were bled 4 hr after this inoculation. Treatment with anti-pro-Th2 cytokine mAbs and isotype control mAbs was then discontinued, but all mice were inoculated o. g. every other day for an additional 5 weeks with MCT/EW. Mice were again followed for decreases in rectal temperature for 1 hour after the last o.g. inoculation (B, right panel). Mice were again bled 4 hours after this o. g. inoculation and total IgE, EW-specific IgG1, and MMCP1 levels were evaluated by ELISA (C). For all figures, * indicates $p<0.05$, as compared to isotype control treated mice.

Figure 2. IL-25, IL-33, and TSLP are all required for development of food allergy in EW + MCT-inoculated mice. A. BALB/c mice, 4-6/group, were fasted for 4 hours and left untreated or inoculated o. g. with 100 µl of MCT on day 0 and day 3. MCT-treated mice were then inoculated o.g. with MCT/EW emulsion every other day for three weeks. Mice were also injected i.p. 12 h before each MCT/EW inoculation with anti-TSLP mAb, anti-IL-25 mAb, anti-IL-33R mAb, a cocktail of anti-TSLP/anti-IL-33/anti-IL-25 mAbs, or with isotype control mAbs 12 h before each MCT/EW dose. Rectal temperatures were determined for the hour after the last o. g. inoculation (B). Mice were bled 4 hours after

this inoculation. IL-4, IL-13, and IFN-γ secretion were evaluated by IVCCA; while serum levels of MMCP1, IgE and IgG1 anti-EW were determined by ELISA (C). In this and other figures, brackets with asterisks indicate a statistically significant ($p<0.05$) different between the groups connected by the bracket.

Figure 3. Established food allergy is suppressed by an anti-pro-Th2 mAb cocktail. A. BALB/c mice were fasted for 4 hours and sensitized with two oral doses of MCT on day 0 and day 3. Then mice were treated with MCT/EW emulsion every other day for four weeks. Mice that developed > 4° C maximum temperature drop were divided into 3 groups of 5 mice per group. All groups were inoculated o. g. with MCT/EW emulsion twice a week for 4 more weeks. The different groups were also injected i.p. with anti-TSLP mAb, with the cocktail of anti-TSLP/anti-IL-33R/anti-IL-25 mAbs, or with isotype control mAbs 12 h before each MCT/EW inoculation. Decreases in rectal temperature were determined for the hour after the last MCT/EW inoculation (B and D). Mice were bled 4 hours after the last o. g. inoculation for determination of serum MMCP1 levels (C) as well as serum IgE levels and IgG1 anti-EW titers (D).

Figure 4. Combined pro-Th2 cytokine blockade is required for effective suppression of established food allergy. A. BALB/c mice were fasted for 4 hours, then inoculated o.g. with 100 µl of MCT on day 0 and day 3. Mice were then kept unimmunized or were inoculated o. g. with MCT/EW emulsion twice a week for four weeks. Mice that developed significant shock (>4° C maximum temperature drop) were divided into 5 groups of 5 mice/group. All groups were then inoculated o. g. with MCT/EW emulsion twice a week for an additional 24 days. Different groups of MCT/EW-immune mice were injected i.p. with the following mAb combinations 12 hours before each o. g. inoculation

with MCT + EW: anti-TSLP + anti-IL-33R mAb; anti-TSLP + anti-IL-25 mAb, anti-IL-25 + anti-IL-33R mAb, anti-TSLP + anti-IL-33R + anti-IL-25 mAb, or isotype control mAbs. Maximal decreases in rectal temperature were determined for the hour following the o.g. inoculation just prior to the initiation of mAb treatment (B, day 0) and for the hour following the o.g. inoculations after 14 and 24 days of mAb treatment (B). Mice were bled 4 h after the day 24 o.g. inoculation to determine levels of IL-4 and IL-13 secretion, MMCP1 response and serum IgE and IgG1 anti-EW levels (C).

Figure 5. Maintenance of increased lamina propria Th2 cell, mast cell (MC), and eosinophil numbers in food allergy is pro-Th2 cytokine-dependent. A. BALB/c mice (4/group) were left untreated (naïve) or were inoculated o.g. with MCT for 3 day, followed by MCT/EW every 4 days for 5 weeks. Following this, mice that had developed a temperature drop of at least 2°C following o.g. inoculation continued to receive o.g. MCT/EW every 4 days for an additional 5 weeks; half of these mice were injected i.p. with anti-TSLP/IL-25/IL-33 mAbs, half with isotype control mAbs, 4 h before each o.g. inoculation. Following the last o.g. inoculation, lamina propria (LP) and mesenteric lymph node (MLN) single cell suspensions were prepared, stained for Th2 cell, ILC2, mast cell, basophil, eosinophil or dendritic cell markers and analyzed for number of each cell type (B) by Coulter counting and flow cytometry.

Supplemental Figure.

Figure S1. Development of hypothermia in response to ingested Ag is IgE-dependent in food-allergic mice. BALB/c mice (4/group) were inoculated o.g. with MCT for 3 days, then with MCT/EW twice a week for 5 weeks, until they developed hypothermia in

response to o.g. inoculation. Mice were then injected i.p. with 500 μg of anti-IgE mAb (EM-95), 500 μg of anti-FcγRIIB/RIII mAb (2.4G2), both mAbs, or isotype control mAbs. One day later, mice were challenged o.g. with MCT/EW and rectal temperatures were followed for the next 60 minutes.

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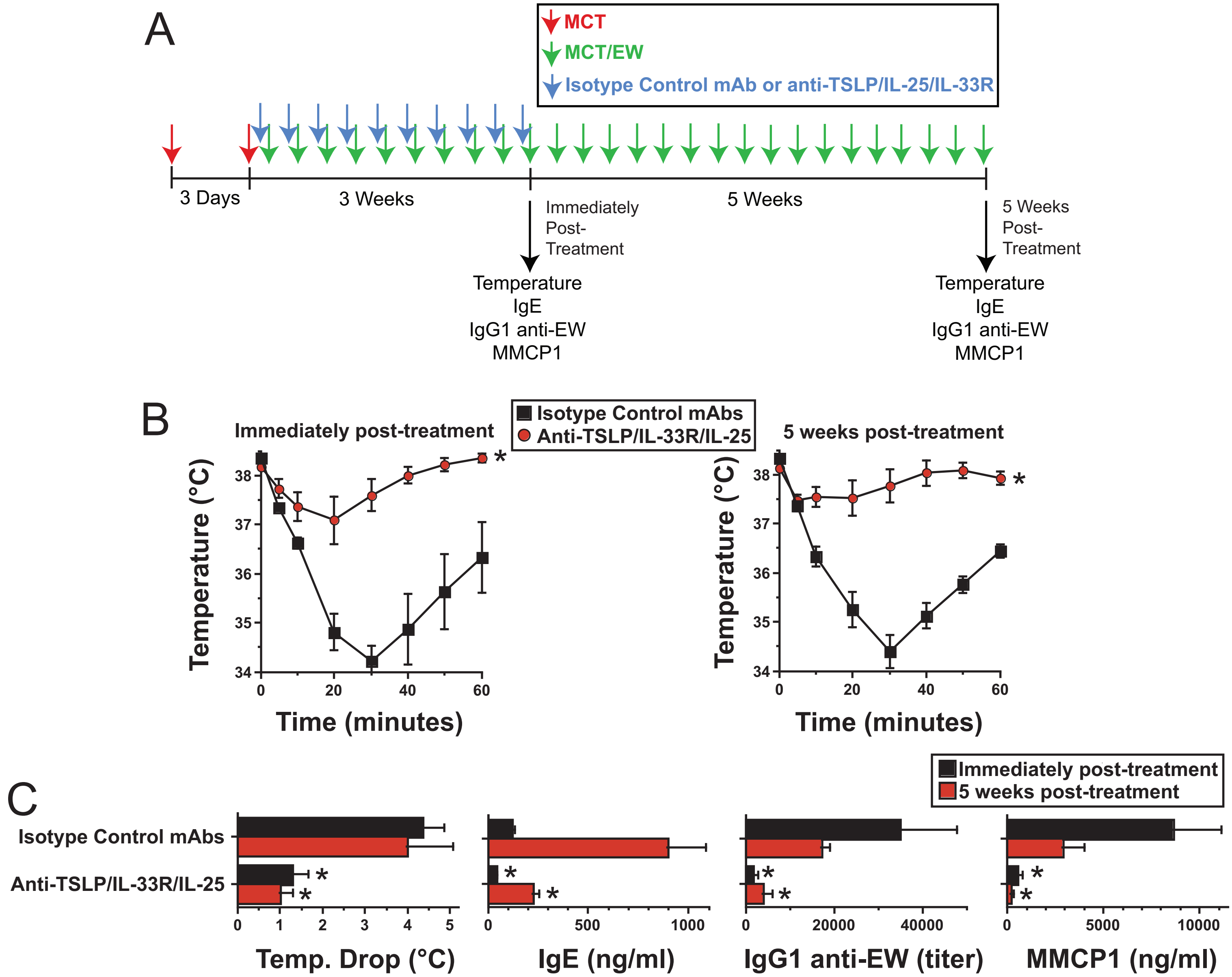

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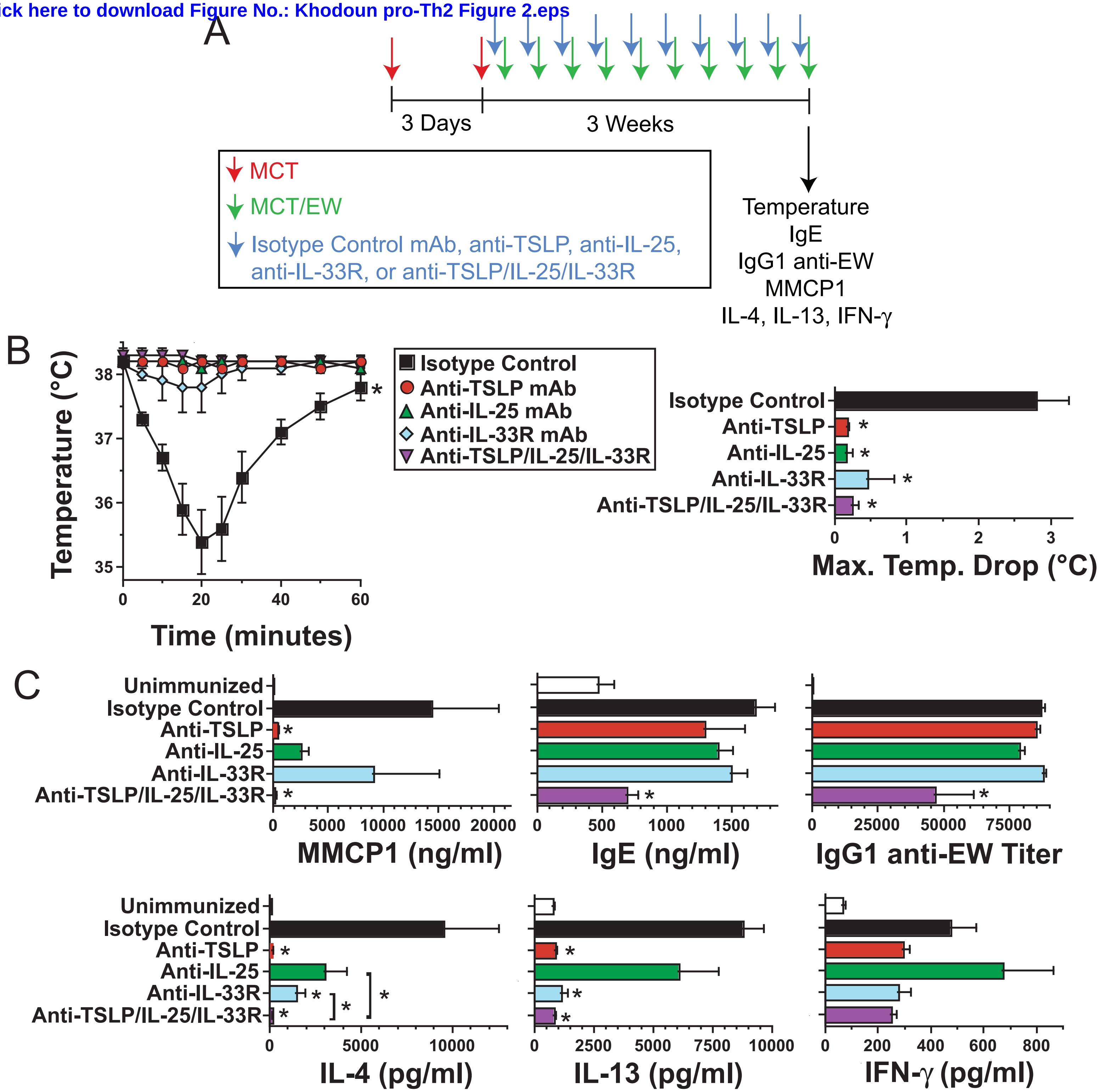

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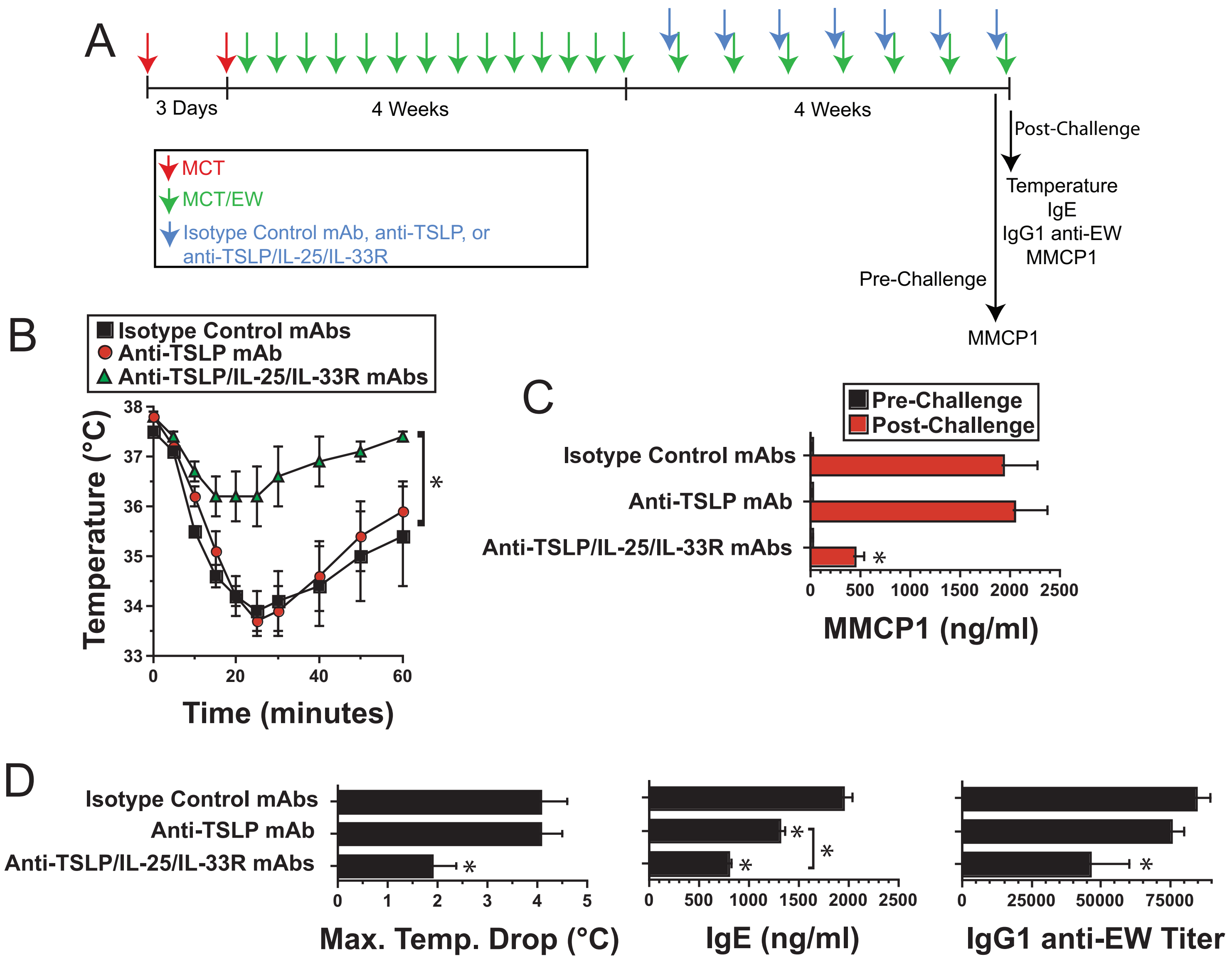

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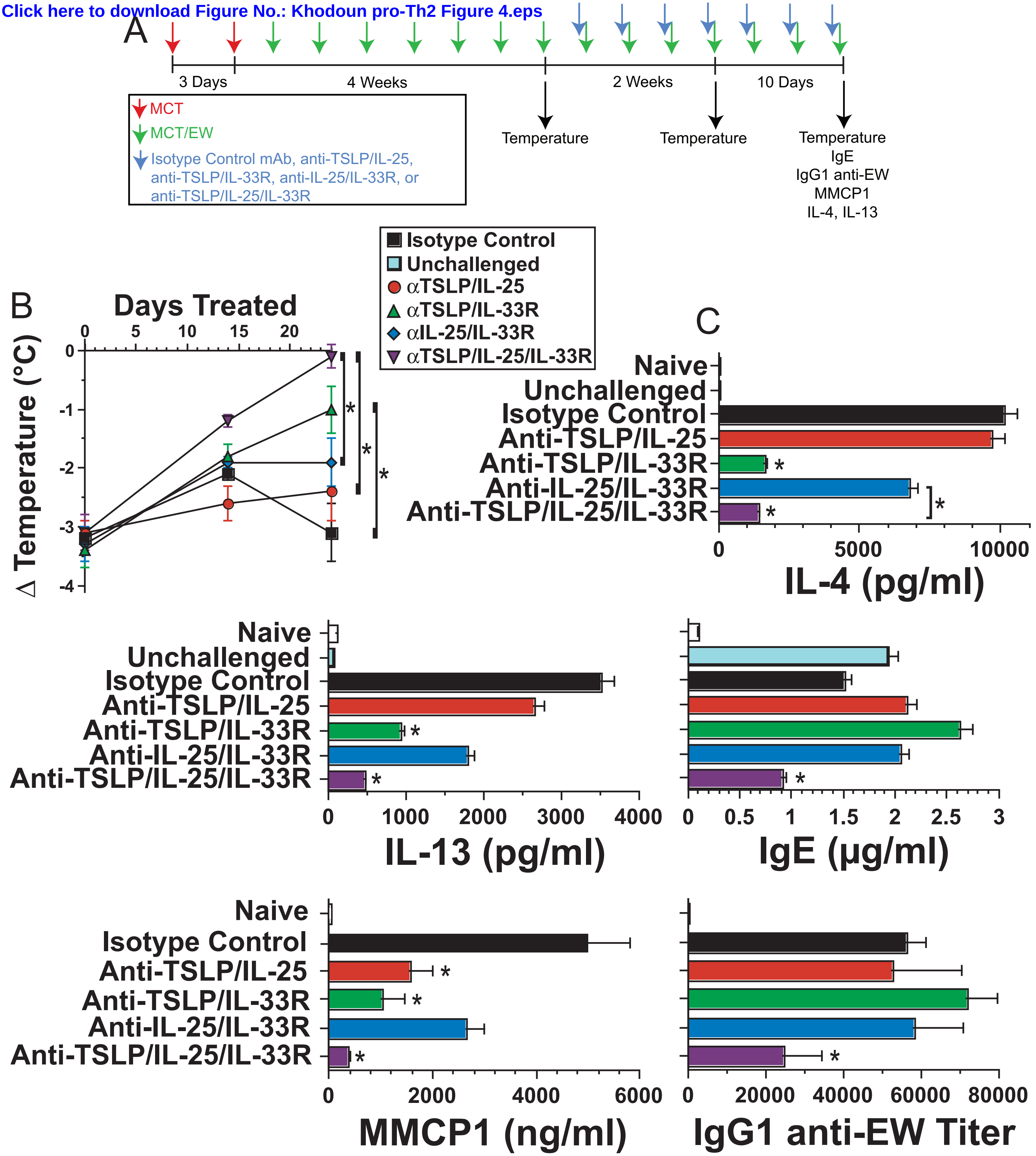

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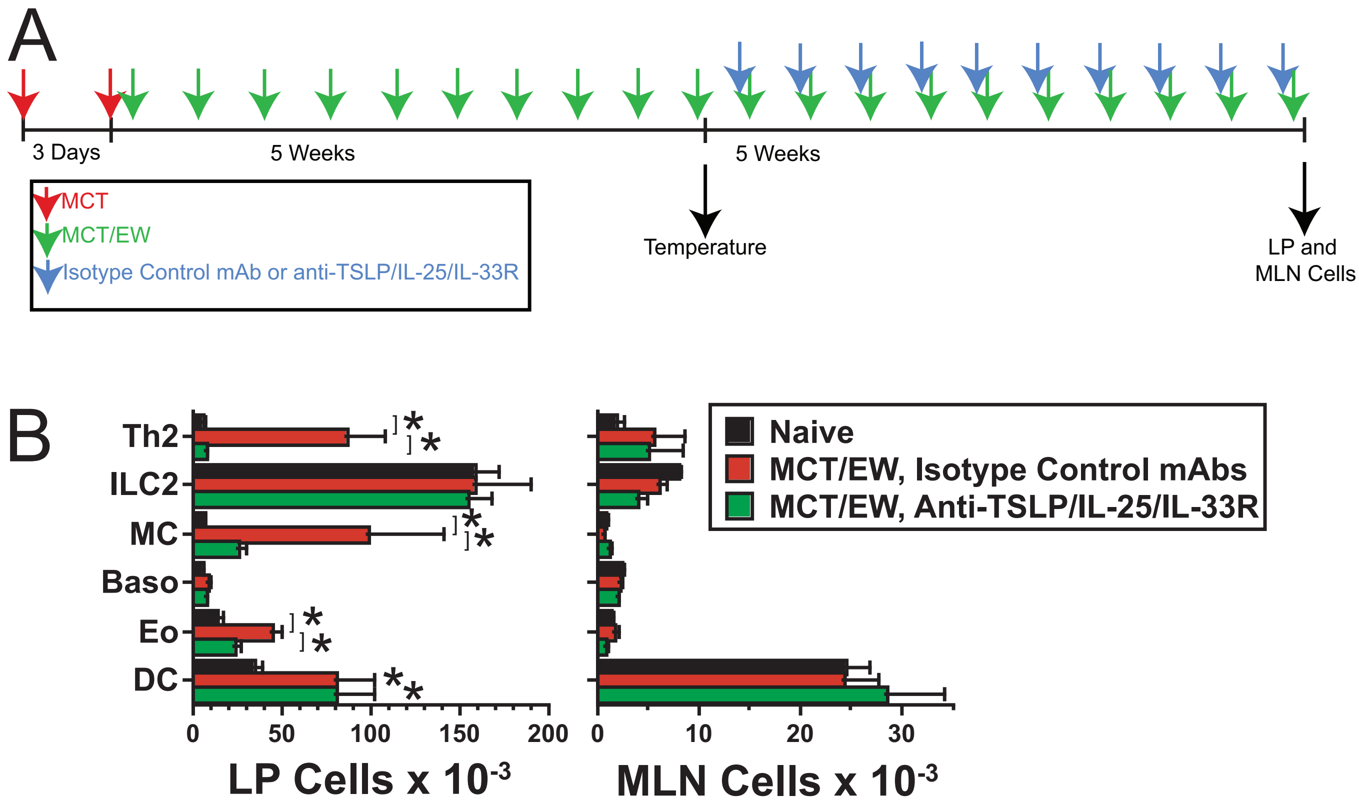

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